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(54) Title: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

(57) Abstract: The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

Methods and Compositions for RNA Interference

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5 GM62534. The United States Government may have certain rights in the invention.

Background of the Invention

“RNA interference”, “post-transcriptional gene silencing”, “quelling” — these different names describe similar effects that result from the overexpression or
10 misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire A (1999) Trends Genet 15:358–363; Sharp PA (1999) Genes Dev 13:139–141; Hunter C (1999) Curr Biol 9:R440–R442; Baulcombe DC (1999) Curr Biol 9:R599–R601; Vaucheret et al. (1998) Plant J 16:651–659). The injection of double-stranded RNA into the nematode *Caenorhabditis elegans*, for example, acts systemically
15 to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire et al. (1998) Nature 391: 806–811; and Montgomery et al. (1998) PNAS 95:15502–15507). RNA interference, commonly referred to as RNAi, offers a way of specifically and potentially inactivating a cloned gene, and is proving a powerful tool for investigating gene function. But the phenomenon is interesting in its own right; the mechanism has been
20 rather mysterious, but recent research — the latest reported by Smardon et al. (2000) Curr Biol 10:169–178 — is beginning to shed light on the nature and evolution of the biological processes that underlie RNAi.

RNAi was discovered when researchers attempting to use the antisense RNA approach to inactivate a *C. elegans* gene found that injection of sense-strand RNA was
25 actually as effective as the antisense RNA at inhibiting gene function. Guo et al. (1995) Cell 81:611–620. Further investigation revealed that the active agent was modest amounts of double-stranded RNA that contaminate *in vitro* RNA preparations. Researchers quickly determined the ‘rules’ and effects of RNAi. Exon sequences are required, whereas introns and promoter sequences, while ineffective, do not appear to compromise RNAi (though
30 there may be gene-specific exceptions to this rule). RNAi acts systemically — injection into one tissue inhibits gene function in cells throughout the animal. The results of a variety of experiments, in *C. elegans* and other organisms, indicate that RNAi acts to destabilize cellular RNA after RNA processing.

The potency of RNAi inspired Timmons and Fire (1998 Nature 395: 854) to do a simple experiment that produced an astonishing result. They fed to nematodes bacteria that had been engineered to express double-stranded RNA corresponding to the *C. elegans unc-22* gene. Amazingly, these nematodes developed a phenotype similar to that of *unc-22* mutants that was dependent on their food source. The ability to conditionally expose large numbers of nematodes to gene-specific double-stranded RNA formed the basis for a very powerful screen to select for RNAi-defective *C. elegans* mutants and then to identify the corresponding genes.

Double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous in vivo contexts including *Drosophila*, *Caenorhabditis elegans*, planaria, hydra, trypanosomes, fungi and plants. However, the ability to recapitulate this phenomenon in higher eukaryotes, particularly mammalian cells, has not been accomplished in the art. Nor has the prior art demonstrated that this phenomena can be observed in cultured eukaryotes cells.

15 Summary of the Invention

One aspect of the present invention provides a method for attenuating expression of a target gene in a non-embryonic cell suspended in culture, comprising introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

Another aspect of the present invention provides a method for attenuating expression of a target gene in a mammalian cell, comprising

- (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and
- 25 (ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

In certain embodiments, the cell is suspended in culture; while in other embodiments the cell is in a whole animal, such as a non-human mammal.

In certain preferred embodiments, the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both. For instance, the recombinant gene may encode, for example, a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID No. 2 or 4; or be

defined by a coding sequence hybridizes under wash conditions of 2 x SSC at 22°C to SEQ ID No. 1 or 3. In certain embodiments, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to the Argonaut sequence shown in Figure 24.

5 In certain embodiments, rather than use a heterologous expression construct(s), an endogenous Dicer gene or Argonaut gene can be activated, e.g. by gene activation technology, expression of activated transcription factors or other signal transduction protein, which induces expression of the gene, or by treatment with an endogenous factor which upregulates the level of expression of the protein or inhibits the degradation of the
10 protein.

In certain preferred embodiments, the target gene is an endogenous gene of the cell. In other embodiments, the target gene is an heterologous gene relative to the genome of the cell, such as a pathogen gene, e.g., a viral gene.

In certain embodiments, the cell is treated with an agent that inhibits protein kinase
15 RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKF.

In certain preferred embodiments, the cell is a primate cell, such as a human cell.

In certain embodiments, the dsRNA is at least 50 nucleotides in length, and preferably 400-800 nucleotides in length.

20 Still another aspect of the present invention provides an assay for identifying nucleic acid sequences responsible for conferring a particular phenotype in a cell, comprising

- (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA;
- 25 (ii) introducing the variegated dsRNA library into a culture of target cells, which cells have an activated Dicer activity or Argonaut activity;
- (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.

30

Yet another aspect of the present invention provides a method of conducting a drug discovery business comprising:

- (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
- (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene;
- 5 (iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and
- (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.

The method may include an additional step of establishing a distribution system for
10 distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Another aspect of the present invention provides a method of conducting a target discovery business comprising:

- 15 (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
- (ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and
- (iii). licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

20 Another aspect of the invention provides a method for inhibiting RNAi by inhibiting the expression or activity of an RNAi enzyme. Thus, the subject method may include inhibiting the activity of Dicer and/or the 22-mer RNA.

Still another aspect relates to the a method for altering the specificity of an RNAi by modifying the sequence of the RNA component of the RNAi enzyme.

25 Another aspect of the invention relates to purified or semi-purified preparations of the RNAi enzyme or components thereof. In certain embodiments, the preparations are used for identifying compounds, especially small organic molecules, which inhibit or potentiate the RNAi activity. Small molecule inhibitors, for example, can be used to inhibit dsRNA responses in cells which are purposefully being transfected with a virus
30 which produces double stranded RNA.

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary

RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA construct may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that

5 nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment

10 algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

15 **Brief Description of the Drawings**

Figure 1: RNAi in S2 cells. a, *Drosophila* S2 cells were transfected with a plasmid that directs *lacZ* expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or *lacZ*, or with no dsRNA, as indicated. b, S2 cells were co-transfected with a plasmid that directs expression of a GFP-US9 fusion protein

20 (12) and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. c, Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

25 Figure 2: RNAi *in vitro*. a, Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. b, Transcripts were incubated in an extract of S2

30 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min.

35 c, Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final

600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Figure 3: Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20- μ l aliquots, as indicated, were pre-incubated with 1 mM CaCl_2 and 5 mM EGTA, 1 mM CaCl_2 , 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM CaCl_2 and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30-min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 μ g) was added to all samples. Time points were at 0 and 30 min.

Figure 4: The RISC contains a potential guide RNA. a, Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E* mRNA. b, Soluble *cyclin-E*-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the *lacZ*, control substrate (upper panel) or the *cyclin E* substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labelled transcript derived from sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Figure 5: Generation of 22mers and degradation of mRNA are carried out by distinct enzymatic complexes. A. Extracts prepared either from 0-12 hour *Drosophila* embryos or *Drosophila* S2 cells (see Methods) were incubated 0, 15, 30, or 60 minutes (left to right) with a uniformly-labeled double-stranded RNA corresponding to the first 500 nucleotides of the *Drosophila cyclin E* coding region. M indicates a marker prepared by *in vitro* transcription of a synthetic template. The template was designed to yield a 22 nucleotide transcript. The doublet most probably results from improper initiation at the +1 position. B. Whole-cell extracts were prepared from S2 cells that had been transfected with a dsRNA corresponding to the first 500 nt. of the luciferase coding region. S10 extracts were spun at 30,000xg for 20 minutes which represents our standard RISC extract⁶. S100 extracts were prepared by further centrifugation of S10 extracts for 60 minutes at 100,000xg. Assays for mRNA degradation were carried out as described previously⁶ for 0,30 or 60 minutes (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded *cyclin E* mRNA, as indicated. C. S10 or S100 extracts were incubated with *cyclin E* dsRNAs for 0, 60 or 120 minutes (L to R).

Figure 6: Production of 22mers by recombinant CG4792/Dicer. A. *Drosophila* S2 cells were transfected with plasmids that direct the expression of T7-epitope tagged

versions of Drosha, CG4792/Dicer-1 and Homeless. Tagged proteins were purified from cell lysates by immunoprecipitation and were incubated with *cyclin E* dsRNA. For comparison, reactions were also performed in *Drosophila* embryo and S2 cell extracts. As a negative control, immunoprecipitates were prepared from cells transfected with a β -galactosidase expression vector. Pairs of lanes show reactions performed for 0 or 60 minutes. The synthetic marker (M) is as described in the legend to Figure 1. B. Diagrammatic representations of the domain structures of CG4792/Dicer-1, Drosha and Homeless are shown. C. Immunoprecipitates were prepared from detergent lysates of S2 cells using an antiserum raised against the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792). As controls, similar preparations were made with a pre-immune serum and with an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with an ~500 nt. fragment of *Drosophila* cyclin E are shown. For comparison, an incubation of the substrate in *Drosophila* embryo extract was electrophoresed in parallel. D. Dicer immunoprecipitates were incubated with dsRNA substrates in the presence or absence of ATP. For comparison, the same substrate was incubated with S2 extracts that either contained added ATP or that were depleted of ATP using glucose and hexokinase (see methods). E. *Drosophila* S2 cells were transfected with uniformly, ³²P-labelled dsRNA corresponding to the first 500 nt. of GFP. RISC complex was affinity purified using a histidine-tagged version of D.m. Ago-2, a recently identified component of the RISC complex (Hammond et al., in prep). RISC was isolated either under conditions in which it remains ribosome associated (ls, low salt) or under conditions that extract it from the ribosome in a soluble form (hs, high salt)⁶. For comparison, the spectrum of labelled RNAs in the total lysate is shown. F. Guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate are compared to guide RNAs present in a affinity-purified RISC complex. These precisely comigrate on a gel that has single-nucleotide resolution. The lane labelled control is an affinity selection for RISC from cell that had been transfected with labeled dsRNA but not with the epitope-tagged D.m. Ago-2.

Figure 7: Dicer participates in RNAi. A. *Drosophila* S2 cells were transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or with a control dsRNA corresponding to murine caspase 9. Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduced activity in lysates by 7.4-fold. B. The Dicer-1 antiserum (CG4792) was used to prepare immunoprecipitates from S2 cells that had been treated as described above. Dicer dsRNA reduced the activity of Dicer-1 in this assay by 6.2-fold. C. Cells that had been transfected two days previously with either mouse caspase 9 dsRNA or with Dicer dsRNA were cotransfected with a GFP expression plasmid and either control, luciferase dsRNA or GFP dsRNA.

Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmid plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase 9) or Dicer dsRNAs.

- 5 Figure 8: Dicer is an evolutionarily conserved ribonuclease. A. A model for production of 22mers by Dicer. Based upon the proposed mechanism of action of Ribonuclease III, we propose that Dicer acts on its substrate as a dimer. The positioning of the two ribonuclease domains (RIIIa and RIIIb) within the enzyme would thus determine the size of the cleavage product. An equally plausible alternative model could
10 be derived in which the RIIIa and RIIIb domains of each Dicer enzyme would cleave in concert at a single position. In this model, the size of the cleavage product would be determined by interaction between two neighboring Dicer enzymes. B. Comparison of the domain structures of potential Dicer homologs in various organisms (*Drosophila* - CG4792, CG6493, *C. elegans* - K12H4.8, *Arabidopsis* - CARPEL FACTORY²⁴,
15 T25K16.4, AC012328_1, human Helicase-MOI²⁵ and *S. pombe* - YC9A_SCHPO). The ZAP domains were identified both by analysis of individual sequences with Pfam²⁷ and by Psi-blast²⁸ searches. The ZAP domain in the putative *S. pombe* Dicer is not detected by PFAM but is identified by Psi-Blast and is thus shown in a different color. For comparison, a domain structure of the RDE1/QDE2/ARGONAUTE family is shown. It
20 should be noted that the ZAP domains are more similar within each of the Dicer and ARGONAUTE families than they are between the two groups. C. An alignment of the ZAP domains in selected Dicer and Argonaute family members is shown. The alignment was produced using ClustalW.

Figure 9: Purification strategy for RISC. (second step in RNAi model).

- 25 Figure 10: Fractionation of RISC activity over sizing column. Activity fractionates as 500KD complex. Also, antibody to dm argonaute 2 cofractionates with activity.

Figure 11-13: Fractionation of RISC over monoS, monoQ, Hydroxyapatite columns. Dm argonaute 2 protein also cofractionates.

Figure 14: Alignment of dm argonaute 2 with other family members.

- 30 Figure 15: Confirmation of dm argonaute 2. S2 cells were transfected with labeled dsRNA and His tagged argonaute. Argonaute was isolated on nickel agarose and RNA component was identified on 15% acrylamide gel.

Figure 16: S2 cell and embryo extracts were assayed for 22mer generating activity.

Figure 17: RISC can be separated from 22mer generating activity (dicer). Spinning extracts (S100) can clear RISC activity from supernatant (left panel) however, S100 spins still contain dicer activity (right panel).

Figure 18: Dicer is specific for dsRNA and prefers longer substrates.

5 Figure 19: Dicer was fractionated over several columns.

Figure 20: Identification of dicer as enzyme which can process dsRNA into 22mers. Various RNaseIII family members were expressed with n terminal tags, immunoprecipitated, and assayed for 22mer generating activity (left panel). In right panel, antibodies to dicer could also precipitate 22mer generating activity.

10 Figure 21: Dicer requires ATP.

Figure 22: Dicer produces RNAs that are the same size as RNAs present in RISC.

Figure 23: Human dicer homolog when expressed and immunoprecipitated has 22mer generating activity.

15 Figure 24: Sequence of dm argonaute 2. Peptides identified by microsequencing are shown in underline.

Figure 25: Molecular characterization of dm argonaute 2. The presence of an intron in coding sequence was determined by northern blotting using intron probe. This results in a different 5' reading frame than that published genome sequence. Number of polyglutamine repeats was determined by genomic PCR.

20 Figure 26: Dicer activity can be created in human cells by expression of human dicer gene. Host cell was 293. Crude extracts had dicer activity, while activity was absent from untransfected cells. Activity is not dissimilar to that seen in drosophila embryo extracts..

25 Figure 27: An ~500 nt. fragment of the gene that is to be silenced (X) is inserted into the modified vector as a stable direct repeat using standard cloning procedures. Treatment with commercially available cre recombinase reverses sequences within the loxP sites (L) to create an inverted repeat. This can be stably maintained and amplified in an sbc mutant bacterial strain (DL759). Transcription in vivo from the promoter of choice (P) yields a hairpin RNA that causes silencing. A zeocin resistance marker is included to
30 insure maintenance of the direct and inverted repeat structures; however this is non-essential in vivo and could be removed by pre-mRNA splicing if desired. Smith, N. A. *et al.* Total silencing by intron-spliced hairpin RNAs. *Nature* 407, 319-20 (2000).

Detailed Description of the Certain Preferred Embodiments

I. Overview

5 The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene).

10 A significant aspect to certain embodiments of the present invention relates to the demonstration in the present application that RNAi can in fact be accomplished in cultured cells, rather than whole organisms as described in the art.

Another salient feature of the present invention concerns the ability to carry out RNAi in higher eukaryotes, particularly in non-oocytic cells of mammals, e.g., cells from adult mammals as an example.

15 As described in further detail below, the present invention(s) are based on the discovery that the RNAi phenomenon is mediated by a set of enzyme activities, including an essential RNA component, that are evolutionarily conserved in eukaryotes ranging from plants to mammals.

20 One enzyme contains an essential RNA component. After partial purification, a multi-component nuclease (herein "RISC nuclease") co-fractionates with a discrete, 22-nucleotide RNA species which may confer specificity to the nuclease through homology to the substrate mRNAs. The short RNA molecules are generated by a processing reaction from the longer input dsRNA. Without wishing to be bound by any particular theory, these 22mer guide RNAs may serve as guide sequences that instruct the RISC nuclease to destroy specific mRNAs corresponding to the dsRNA sequences.

25 The appended examples also identify an enzyme, Dicer, that can produce the putative guide RNAs. Dicer is a member of the RNase III family of nucleases that specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi and, as described herein, mammals. The enzyme has a distinctive structure which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to
30 the RDE1/QDE2/ARGONAUTE family, which have been genetically linked to RNAi in lower eukaryotes. Indeed, activation of, or overexpression of Dicer may be sufficient in many cases to permit RNA interference in otherwise non-receptive cells, such as cultured eukaryotic cells, or mammalian (non-oocytic) cells in culture or in whole organisms.

In certain embodiments, the cells can be treated with an agent(s) that inhibits the double-stranded RNA-dependent protein known as PKR (protein kinase RNA-activated). Double stranded RNAs in mammalian cells typically activate protein kinase PKR that phosphorylates and inactivates eIF2a (Fire (1999) Trends Genet 15:358). The ensuing inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles. However, as described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However, in certain instances, it can be desirable to treat the cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKF are specifically contemplated for use in the present method. Likewise, overexpression of or agents which ectopically activate IF2 α can be used.

Thus, the present invention provides a process and compositions for inhibiting expression of a target gene in a cell, especially a mammalian cell. In certain embodiments, the process comprises introduction of RNA (the "dsRNA construct") with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce the dsRNA construct. In preferred embodiments, the method utilizes a cell in which Dicer and/or Argonaute activities are recombinantly expressed or otherwise ectopically activated. This process can be (1) effective in attenuating gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to that it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromosomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to that they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

5 As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, that may optionally include intron sequences that are derived from chromosomal DNA. The term "intron" refers to a DNA
10 sequence present in a given gene that is not translated into protein and is generally found between exons. As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

A "protein coding sequence" or a sequence that "encodes" a particular polypeptide
15 or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA
20 from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences that encode a polypeptide, as the term is typically used, as well as DNA
25 sequences that are transcribed into inhibitory antisense molecules.

The term "loss-of-function", as it refers to genes inhibited by the subject RNAi method, refers a diminishment in the level of expression of a gene when compared to the level in the absense of dsRNA constructs.

The term "expression" with respect to a gene sequence refers to transcription of the
30 gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably
35 herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur

in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a dsRNA construct.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein.

A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control sequences can be directly or indirectly regulated by the target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "immortalized cells" refers to cells that have been altered via chemical, genetic, and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

III. Exemplary embodiments of Isolation Method

One aspect of the invention provides a method for potentiating RNAi by induction or ectopic activation of an RNAi enzyme in a cell (in vivo or in vitro) or cell-free mixtures. In preferred embodiments, the RNAi activity is activated or added to a mammalian cell, e.g., a human cell, which cell may be provided in vitro or as part of a whole organism. In other embodiments, the subject method is carried out using eukaryotic cells generally (except for oocytes) in culture. For instance, the Dicer enzyme may be activated by virtue of being recombinantly expressed or it may be activated by use of an agent which (i) induces expression of the endogenous gene, (ii) stabilizes the protein from degradation, and/or (iii) allosterically modifies the enzyme to increase its activity (by altering its Kcat, Km or both).

A. Dicer and Argonaut Activities

In certain embodiment, at least one of the activated RNAi enzymes is Dicer, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Dicer. As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to SEQ ID No. 2 or 4, and/or which can be encoded by a nucleic acid which hybridizes under wash conditions of 2 x SSC at 22°C, and more preferably 0.2 x SSC at 65°C, to a nucleotide represented by SEQ ID No. 1 or 3. Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Dicer has been recombinantly expressed or otherwise ectopically activated.

In certain embodiment, at least one of the activated RNAi enzymes is Argonaut, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Argonaut. As used herein, the term "Argonaut" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to the amino acid sequence shown in Figure 24. Accordingly, the method may comprise

introducing a dsRNA construct into a cell in which Argonaut has been recombinantly expressed or otherwise ectopically activated.

This invention also provides expression vectors containing a nucleic acid encoding a Dicer or Argonaut polypeptides, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject Dicer or Argonaut proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding Dicer or Argonaut polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

The recombinant Dicer or Argonaut genes can be produced by ligating nucleic acid encoding a Dicer or Argonaut polypeptide into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject Dicer or Argonaut polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a Dicer or Argonaut polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived

plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression
5 vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance
10 markers such as ampicillin can be used. In an illustrative embodiment, a Dicer or Argonaut polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of a Dicer or Argonaut gene.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic
15 transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection
20 in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and
25 eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In yet another embodiment, the subject invention provides a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the
30 transcriptional regulatory sequences of an endogenous Dicer or Argonaut gene. For instance, the gene activation construct can replace the endogenous promoter of a Dicer or Argonaut gene with a heterologous promoter, e.g., one which causes constitutive expression of the Dicer or Argonaut gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of Dicer or Argonaut. A
35 variety of different formats for the gene activation constructs are available. See, for

example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous Dicer or Argonaut gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic Dicer or Argonaut gene upon recombination of the gene activation construct. For use in generating cultures of Dicer or Argonaut producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native Dicer or Argonaut gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous Dicer or Argonaut gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*,

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 5 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a
10 positive control element, e.g., to inhibit expression of the targeted gene.

B. Cell/Organism

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). The dsRNA construct may be
15 synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For generating double stranded transcripts from a transgene in vivo, a regulatory region may be used to transcribe the RNA strand (or strands).

Furthermore, genetic manipulation becomes possible in organisms that are not
20 classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

25 The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast
30 morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro,
35 tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry,

blueberry, cacao, cherry, coconut, cranberry, date, faJoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, 5 carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

Invertebrate animals include nematodes, other worms, drosophila, and other 10 insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfllichonema, Toxocara, Uncinaria) and those that infect plants (e.g., B ursaphalenchus, 15 Criconerriella, Diilylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynychus, Tylenchus, and Xiphinerna). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or 20 pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, 25 osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

C. Targeted Genes

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. 30 Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA 35 or translation of target protein.

“Inhibition of gene expression” refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. “Specificity” refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracycline.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABLI, BCL1, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC,

MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA 1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and WTI); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

D. dsRNA constructs

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNA construct may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The dsRNA construct may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower

doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

dsRNA constructs containing a nucleotide sequences identical to a portion of the
5 target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 199 1, and references cited therein) and calculating the percent
10 difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined
15 functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be, for example, at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

20 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The dsRNA construct may be synthesized either in vivo or in vitro. Endogenous
25 RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the dsRNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue,
30 or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The dsRNA construct may be chemically or enzymatically synthesized by manual or
35 automated reactions. The dsRNA construct may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and

production of an expression construct are known in the art^{32,33,34} (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be
5 purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography or a combination thereof. Alternatively, the dsRNA construct may be used with no or a minimum of purification to avoid losses due to sample processing. The dsRNA construct may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or
10 stabilization of the duplex strands.

Physical methods of introducing nucleic acids include injection of a solution containing the dsRNA construct, bombardment by particles covered by the dsRNA construct, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the dsRNA construct. A viral construct packaged into a viral
15 particle would accomplish both efficient introduction of an expression construct into the cell and transcription of dsRNA construct encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the dsRNA construct may be introduced along with components that
20 perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

E. Illustrative Uses

25 One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing
30 the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources,
35 including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different

organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example, tissue culture cells derived from mammals, especially primates, and most preferably humans.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the

duplex RNA can be introduced to the organism or cell, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

IV. Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1: An RNA-directed nuclease mediates RNAi gene silencing

In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner^{1,7}. These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defence, modulate transposition or regulate gene expression^{1, 6, 8-10}. We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila*^{11, 12}, the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in β -galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (*CD8*) (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but transfection with the *cyclin E* dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100

nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect.

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed
5 diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (Fig. 1c). The modest reduction in *fizzy* mRNA
10 levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low^{14, 15}. These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional
15 levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in refs 1 and 6). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts
20 prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely, lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have
25 termed this enzyme RISC (RNA-induced silencing complex). Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the
30 degradation process¹⁶. In addition, our ability to create an extract that targets *lacZ* *in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig.
35 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-

nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the *cyclin E* mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected^{17, 18}. Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vivo* (Fig. 2b). In the *in vitro* system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing¹⁹. In accord with this idea, pre-treatment of extracts with a Ca²⁺-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNase I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced¹². To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our

activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and
5 ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on
10 northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

15 RNA interference allows an adaptive defence against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others¹², is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the
20 post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants¹² and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in
25 diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodelling and transcriptional repression^{20, 21}. It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodelling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences.

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Methods

Cell culture and RNA methods S2 (ref. 22) cells were cultured at 27 °C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation²³.
35 Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with

a vector that directs expression of a green fluorescent protein (GFP)-US9 fusion protein¹³. These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 $\mu\text{g ml}^{-1}$. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNase III (a gift from A. Nicholson). Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β -mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units ml^{-1} of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000g for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $\text{Mg}(\text{OAc})_2$, 3 mM EGTA, 2 mM CaCl_2 , 1 mM DTT. Typically, 5 μl extract was used in a 10 μl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation Extracts were centrifuged at 200,000g for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM MgCl_2 and 300 mM KOAc. The extracted material was spun at 100,000g for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM MgCl_2). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM NaPO_4 pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1 SSC at 37–45 $^{\circ}\text{C}$.

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Example 2: Role for a bidentate ribonuclease in the initiation step of RNA interference

- 25 Genetic approaches in worms, fungi and plants have identified a group of proteins that are essential for double-stranded RNA-induced gene silencing. Among these are ARGONAUTE family members (e.g. RDE1, QDE2)^{9,10,30}, recQ-family helicases (MUT-7, QDE3)^{11,12}, and RNA-dependent RNA polymerases (e.g. EGO-1, QDE1, SGS2/SDE1)¹³⁻¹⁶. While potential roles have been proposed, none of these genes has been assigned a
- 30 definitive function in the silencing process. Biochemical studies have suggested that PTGS is accomplished by a multicomponent nuclease that targets mRNAs for degradation^{6,8,17}. We have shown that the specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate⁶. Originally identified in plants that were actively silencing transgenes⁷, these ~22 nt. RNAs

have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos⁸. Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (Fig. 5a). With the goal of understanding the mechanism of post-transcriptional gene silencing, we have undertaken both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of a nuclease, RISC, that is an effector of RNA interference. See Example 1. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated *in vivo* by transfection with dsRNA. We first sought to determine whether the RISC enzyme and the enzyme that initiates RNAi via processing of dsRNA into 22mers are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000xg for 60 min.) while the activity that produces 22mers remained in the supernatant (Fig. 5b,c). This simple fractionation indicated that RISC and the 22mer-generating activity are separable and thus distinct enzymes. However, it seems likely that they might interact at some point during the silencing process.

RNAse III family members are among the few nucleases that show specificity for double-stranded RNA¹⁸. Analysis of the *Drosophila* and *C. elegans* genomes reveals several types of RNAse III enzymes. First is the canonical RNAse III which contains a single RNAse III signature motif and a double-stranded RNA binding domain (dsRBD; e.g. RNC_CAEEL). Second is a class represented by Drosha¹⁹, a *Drosophila* enzyme that contains two RNAse III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNAse III signatures and an amino terminal helicase domain (e.g. *Drosophila* CG4792, CG6493, *C. elegans* K12H4.8), and these had previously been proposed by Bass as candidate RNAi nucleases²⁰. Representatives of all three classes were tested for the ability to produce discrete, ~22 nt. RNAs from dsRNA substrates.

Partial digestion of a 500 nt. cyclin E dsRNA with purified, bacterial RNAse III produced a smear of products while nearly complete digestion produced a heterogeneous group of ~11-17 nucleotide RNAs (not shown). In order to test the dual-RNAse III enzymes, we prepared T7 epitope-tagged versions of Drosha and CG4792. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody-agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded ~22 nt. fragments similar to those produced in either S2 or embryo extracts (Fig. 6a). Neither activity in extract nor activity in immunoprecipitates depended on the sequence of the RNA substrate since dsRNAs derived from several genes were processed equivalently (see Supplement 1). Negative results were obtained with Drosha and with immunoprecipitates of a DEXH box helicase (Homeless²¹; see Fig 6a,b). Western blotting

confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplement 2). Thus, we conclude that CG4792 may carry out the initiation step of RNA interference by producing ~22 nt. guide sequences from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells, and in adult flies, consistent with the presence of functional RNAi machinery in all of these contexts (see Supplement 3).

The possibility that Dicer might be the nuclease responsible for the production of guide RNAs from dsRNAs prompted us to raise an antiserum directed against the carboxy-terminus of the Dicer protein (Dicer-1, CG4792). This antiserum could immunoprecipitate a nuclease activity from either *Drosophila* embryo extracts or from S2 cell lysates that produced ~22 nt. RNAs from dsRNA substrates (Fig. 6C). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely comigrate with 22mers that are produced in extract and with 22mers that are associated with the RISC enzyme (Fig. 6 D,F). It had previously been shown that the enzyme that produced guide RNAs in *Drosophila* embryo extracts was ATP-dependent⁸. Depletion of this cofactor resulted in an ~6-fold lower rate of dsRNA cleavage and in the production of RNAs with a slightly lower mobility. Of interest was the fact that both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22mers (Fig. 6D). We do not observe the accumulation of lower mobility products in these cases, although we do routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is a quite unusual property. We hypothesize that this requirement could indicate that the enzyme may act processively on the dsRNA, with the helicase domain harnessing the energy of ATP hydrolysis both for unwinding guide RNAs and for translocation along the substrate.

Efficient induction of RNA interference in *C. elegans* and in *Drosophila* has several requirements. For example, the initiating RNA must be double-stranded, and it must be several hundred nucleotides in length. To determine whether these requirements are dictated by Dicer, we characterized the ability of extracts and of immunoprecipitated enzyme to digest various RNA substrates. Dicer was inactive against single stranded RNAs regardless of length (see Supplement 4). The enzyme could digest both 200 and 500 nucleotide dsRNAs but was significantly less active with shorter substrates (see Supplement 4). Double-stranded RNAs as short as 35 nucleotides could be cut by the enzyme, albeit very inefficiently (data not shown). In contrast, *E. coli* RNase III could digest to completion dsRNAs of 35 or 22 nucleotides (not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to but not wholly determine the size dependence of RNAi.

To determine whether the Dicer enzyme indeed played a role in RNAi *in vivo*, we sought to deplete Dicer activity from S2 cells and test the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a mixture of dsRNAs homologous to the two *Drosophila* Dicer genes (CG4792 and CG6493) resulted in an ~6-7 fold reduction of Dicer activity either in whole cell lysates or in Dicer-1 immunoprecipitates (Fig. 7A,B). Transfection with a control dsRNA (murine caspase 9) had no effect. Qualitatively similar results were seen if Dicer was examined by Northern blotting (not shown). Depletion of Dicer in this manner substantially compromised the ability of cells to silence subsequently an exogenous, GFP transgene by RNAi (Fig. 7C). These results indicate that Dicer is involved in RNAi *in vivo*. The lack of complete inhibition of silencing could result from an incomplete suppression of Dicer (which is itself required for RNAi) or could indicate that *in vivo*, guide RNAs can be produced by more than one mechanism (e.g. through the action of RNA-dependent RNA polymerases).

Our results indicate that the process of RNA interference can be divided into at least two distinct steps. According to this model, initiation of PTGS would occur upon processing of a double-stranded RNA by Dicer into ~22 nucleotide guide sequences, although we cannot formally exclude the possibility that another, Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have demonstrated that ³²P-labeled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22 mers (Fig. 7E). However, we cannot exclude the possibility that RNA-dependent RNA polymerases might amplify 22mers once they have been generated or provide an alternative method for producing guide RNAs.

The structure of the Dicer enzyme provokes speculation on the mechanism by which the enzyme might produce discretely sized fragments irrespective of the sequence of the dsRNA (see Supplement 1, Fig. 8a). It has been established that bacterial RNase III acts on its substrate as a dimer^{18,22,23}. Similarly, a dimer of Dicer enzymes may be required for cleavage of dsRNAs into ~22 nt. pieces. According to one model, the cleavage interval would be determined by the physical arrangement of the two RNase III domains within Dicer enzyme (Fig. 8a). A plausible alternative model would dictate that cleavage was directed at a single position by the two RIII domains in a single Dicer protein. The 22 nucleotide interval could be dictated by interaction of neighboring Dicer enzymes or by translocation along the mRNA substrate. The presence of an integral helicase domain suggests that the products of Dicer cleavage might be single-stranded 22 mers that are incorporated into the RISC enzyme as such.

A notable feature of the Dicer family is its evolutionary conservation. Homologs are found in *C. elegans* (K12H4.8), *Arabidopsis* (e.g., CARPEL FACTORY²⁴, T25K16.4, AC012328_1), mammals (Helicase-MOI²⁵) and *S. pombe* (YC9A_SCHPO) (Fig 8b, see Supplements 6,7 for sequence comparisons). In fact, the human Dicer family member is

5 capable of generating ~22 nt. RNAs from dsRNA substrates (Supplement 5) suggesting that these structurally similar proteins may all share similar biochemical functions. It has been demonstrated that exogenous dsRNAs can affect gene function in early mouse embryos²⁹, and our results suggest that this regulation may be accomplished by an evolutionarily conserved RNAi machinery.

10 In addition to RNaseIII and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a ZAP domain (Fig 8c)²⁷. This sequence was defined based solely upon its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations in *C. elegans* (Rde-1)⁹ and *Neurospora* (Qde-2)¹⁰. Although the function of this domain is

15 unknown, it is intriguing that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral

20 meristems²⁴. This phenotype and a number of other characteristic features are also shared by *Arabidopsis* ARGONAUTE (*ago1-1*) mutants²⁶ (C. Kidner and R. Martienssen, pers. comm.). These genetic analyses begin to provide evidence that RNAi may be more than a defensive response to unusual RNAs but may also play important roles in the regulation of endogenous genes.

25 With the identification of Dicer as a catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It will be of critical importance to determine whether the conserved family members from other organisms, particularly mammals, also play a role in dsRNA-mediated gene regulation.

30

Methods

Plasmid constructs. A full-length cDNA encoding Droscha was obtained by PCR from an EST sequenced by the Berkeley Drosophila genome project. The *Homeless* clone was a gift from Gillespie and Berg (Univ. Washington). The T7 epitope-tag was added to

35 the amino terminus of each by PCR, and the tagged cDNAs were cloned into pRIP, a retroviral vector designed specifically for expression in insect cells (E. Bernstein,

unpublished). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). Since no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bacmid (BACR23F10; obtained from the BACPAC Resource Center in the Dept. of Human Genetics at the Roswell Park Cancer Institute). Again, during
5 amplification, a T7 epitope tag was added at the amino terminus of the coding sequence. The human Dicer gene was isolated from a cDNA library prepared from HaCaT cells (GJH, unpublished). A T7-tagged version of the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation. *S2 and embryo culture.* S2 cells were
10 cultured at 27°C in 5% CO₂ in Schneider's insect media supplemented with 10% heat inactivated fetal bovine serum (Gemini) and 1% antibiotic-antimycotic solution (Gibco BRL). Cells were harvested for extract preparation at 10x10⁶ cells/ml. The cells were washed 1X in PBS and were resuspended in a hypotonic buffer (10 mM Hepes pH 7.0, 2mM MgCl₂, 6 mM βME) and dounced. Cell lysates were spun 20,000xg for 20 minutes.
15 Extracts were stored at -80°C. *Drosophila* embryos were reared in fly cages by standard methodologies and were collected every 12 hours. The embryos were dechorionated in 50% chlorox bleach and washed thoroughly with distilled water. Lysis buffer (10mM Hepes, 10mM KCl, 1.5 mM MgCl₂, 0.5mM EGTA, 10mM β-glycerophosphate, 1mM DTT, 0.2 mM PMSF) was added to the embryos, and extracts were prepared by
20 homogenization in a tissue grinder. Lysates were spun for two hours at 200,000xg and were frozen at -80°C. LinX-A cells, a highly-transfectable derivative of human 293 cells, (Lin Xie and GJH, unpublished) were maintained in DMEM/10%FCS.

Transfections and immunoprecipitations. S2 cells were transfected using a calcium phosphate procedure essentially as previously described⁶. Transfection rates were ~90%
25 as monitored in controls using an *in situ* β-galactosidase assay. LinX-A cells were also transfected by calcium phosphate co-precipitation. For immunoprecipitations, cells (~5x10⁶ per IP) were transfected with various clones and lysed three days later in IP buffer (125mM KOAc, 1mM MgOAc, 1mM CaCl₂, 5mM EGTA, 20mM Hepes pH 7.0, 1mM DTT, 1% NP-40 plus Complete protease inhibitors (Roche)). Lysates were spun for 10
30 minutes at 14,000xg and supernatants were added to T7 antibody-agarose beads (Novagen). Antibody binding proceeded for 4 hours at 4°C. Beads were centrifuged and washed in lysis buffer three times, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a KLH-conjugated peptide corresponding to the C-terminal 8 amino acids of *Drosophila* Dicer-1 (CG4792).

35 Cleavage reactions. *RNA preparation.* Templates to be transcribed into dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter

sequence. RNAs were produced using Riboprobe (Promega) kits and were uniformly labeling during the transcription reaction with ^{32}P -UTP. Single-stranded RNAs were purified from 1% agarose gels. *dsRNA cleavage*. Five microliters of embryo or S2 extracts were incubated for one hour at 30°C with dsRNA in a reaction containing 20mM Hepes pH 7.0, 2mM MgOAc, 2mM DTT, 1mM ATP and 5% Supersin (Ambion). Immunoprecipitates were treated similarly except that a minimal volume of reaction buffer (including ATP and Supersin) and dsRNA were added to beads that had been washed in reaction buffer (see above). For ATP depletion, *Drosophila* embryo extracts were incubated for 20 minutes at 30°C with 2mM glucose and 0.375 U of hexokinase (Roche) prior to the addition of dsRNA.

Northern and Western analysis. Total RNA was prepared from *Drosophila* embryos (0-12 hour), from adult flies, and from S2 cells using Trizol (Lifetech). Messenger RNA was isolated by affinity selection using magnetic oligo-dT beads (Dyna). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For Western analysis, T7-tagged proteins were immunoprecipitated from whole cell lysates in IP buffer using anti-T7-antibody-agarose conjugates. Proteins were released from the beads by boiling in Laemmli buffer and were separated by electrophoresis on 8% SDS PAGE. Following transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer. *Drosophila* S2 cells were transfected either with a dsRNA corresponding to mouse caspase 9 or with a mixture of two dsRNAs corresponding to *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as previously described⁶. Cells were assayed for Dicer activity or fluorescence three days after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS cell sorter after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase 9 dsRNA.

30

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Example 3: A simplified method for the creation of hairpin constructs for RNA interference.

In numerous model organisms, double stranded RNAs have been shown to cause
5 effective and specific suppression of gene function (ref. 1). This response, termed RNA
interference or post-transcriptional gene silencing, has evolved into a highly effective
reverse genetic tool in *C. elegans*, *Drosophila*, plants and numerous other systems. In
these cases, double-stranded RNAs can be introduced by injection, transfection or feeding;
however, in all cases, the response is both transient and systemic. Recently, stable
10 interference with gene expression has been achieved by expression of RNAs that form
snap-back or hairpin structures (refs 2-7). This has the potential not only to allow stable
silencing of gene expression but also inducible silencing as has been observed in
trypanosomes and adult *Drosophila* (refs 2,4,5). The utility of this approach is somewhat
hampered by the difficulties that arise in the construction of bacterial plasmids containing
15 the long inverted repeats that are necessary to provoke silencing. In a recent report, it was
stated that more than 1,000 putative clones were screened to identify the desired construct
(ref 7).

The presence of hairpin structures often induces plasmid rearrangement, in part
due to the *E. coli* *sbc* proteins that recognize and cleave cruciform DNA structures (ref 8).
20 We have developed a method for the construction of hairpins that does not require cloning
of inverted repeats, per se. Instead, the fragment of the gene that is to be silenced is
cloned as a direct repeat, and the inversion is accomplished by treatment with a site-
specific recombinase, either *in vitro* (or potentially *in vivo*) (see Fig 29). Following
recombination, the inverted repeat structure is stable in a bacterial strain that lacks an
25 intact SBC system (DL759). We have successfully used this strategy to construct
numerous hairpin expression constructs that have been successfully used to provoke gene
silencing in *Drosophila* cells.

Literature Cited in Example 3

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V. Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention
20 described herein. Such equivalents are intended to be encompassed by the following claims.

All of the above-cited references and publications are hereby incorporated by reference.

We Claim:

1. A method for attenuating expression of a target gene in a non-embryonic cell suspended in culture, comprising introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene,
5 wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.
2. A method for attenuating expression of a target gene in a mammalian cell, comprising
10 (i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and
(ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions
15 to a nucleotide sequence of the target gene.
3. The method of claim 2, wherein the cell is suspended in culture.
4. The method of claim 2, wherein the cell is in a whole animal, such as a non-human
20 mammal.
5. The method of claim 1 or 2, wherein is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both.
25
6. The method of claim 5, wherein the recombinant gene encodes a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID No. 2 or 4 or the Argonaut sequence shown in Figure 24.
- 30 7. The method of claim 5, wherein the recombinant gene includes a coding sequence hybridizes under wash conditions of 2 x SSC at 22°C to SEQ ID No. 1 or 3.
8. The method of claim 1 or 2, wherein an endogenous Dicer gene or Argonaut gene is activated.
35
9. The method of claim 1 or 2, wherein the target gene is an endogenous gene of the cell.

10. The method of claim 1 or 2, wherein the target gene is an heterologous gene relative to the genome of the cell, such as a pathogen gene.
- 5 11. The method of claim 1 or 2, wherein the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKF.
- 10 12. The method of claim 1 or 2, wherein the cell is a primate cell, such as a human cell.
13. The method of claim 1 or 2, wherein the dsRNA is at least 50 nucleotides in length.
- 15 14. The method of claim 13, wherein the dsRNA is 400-800 nucleotides in length.
15. The method of claim 13, wherein the dsRNA is 400-800 nucleotides in length.
- 20 16. An assay for identifying nucleic acid sequences responsible for conferring a particular phenotype in a cell, comprising
- (i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA;
- (ii) introducing the variegated dsRNA library into a culture of target cells, which cells have an activated Dicer activity or Argonaut activity;
- 25 (iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.
- 30 17. A method of conducting a drug discovery business comprising:
- (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
- (ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene;
- 35 (iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and

- (iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.
- 18. The method of claim 17, including an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally
5 include establishing a sales group for marketing the pharmaceutical preparation.
- 19. A method of conducting a target discovery business comprising:
 - (i) identifying, by the assay of claim 16, a target gene which provides a phenotypically desirable response when inhibited by RNAi;
 - 10 (ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and
 - (iii). licensing, to a third party, the rights, for further drug development of inhibitors of the target gene.

15

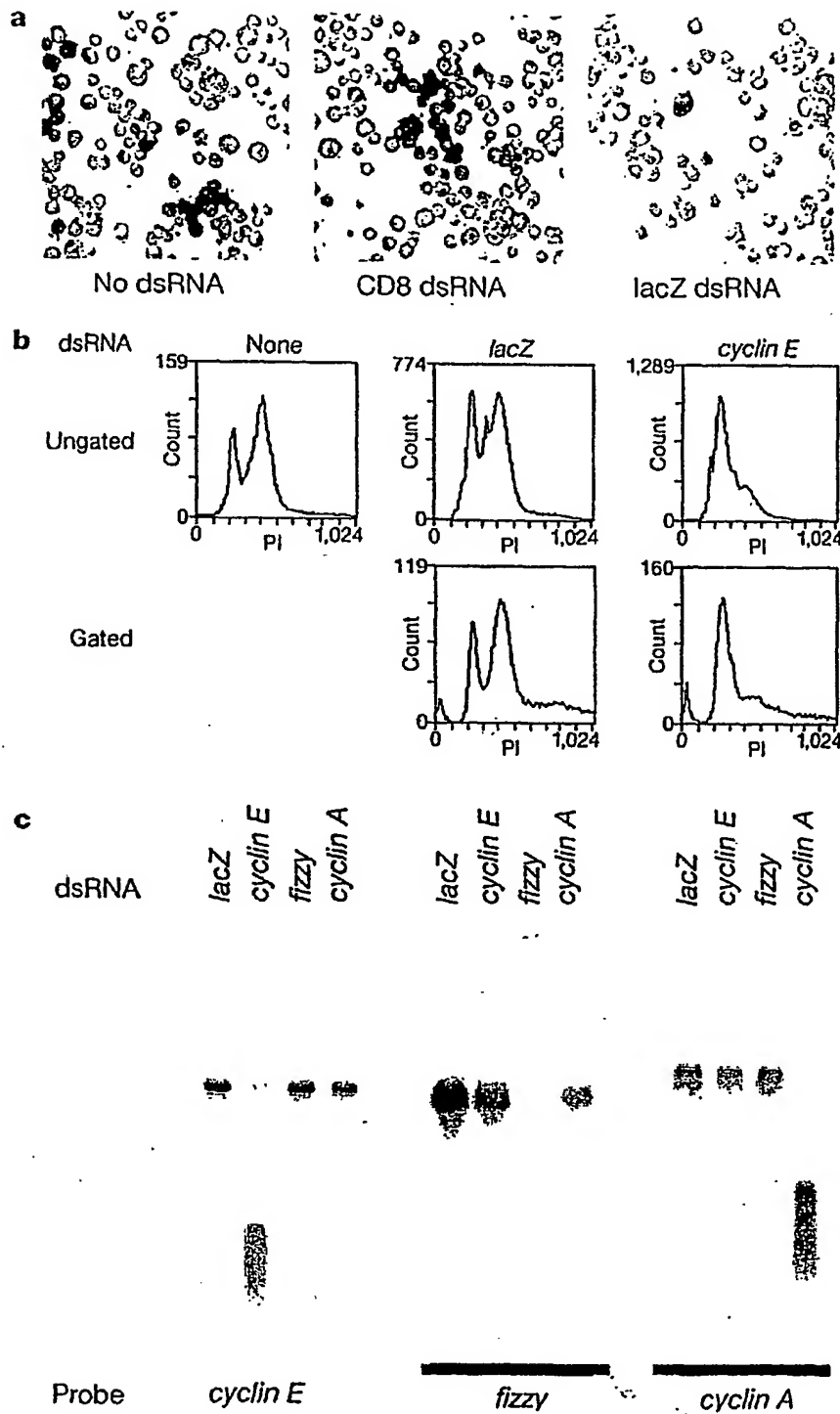


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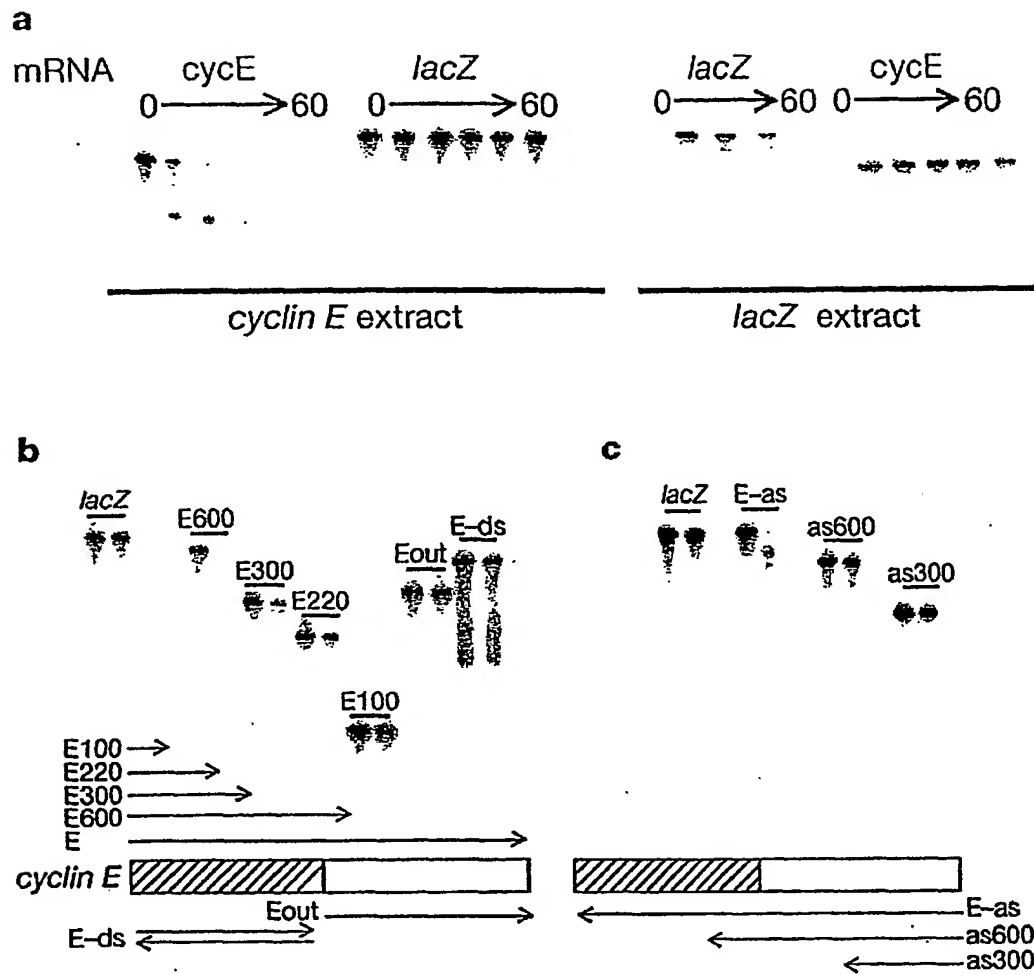


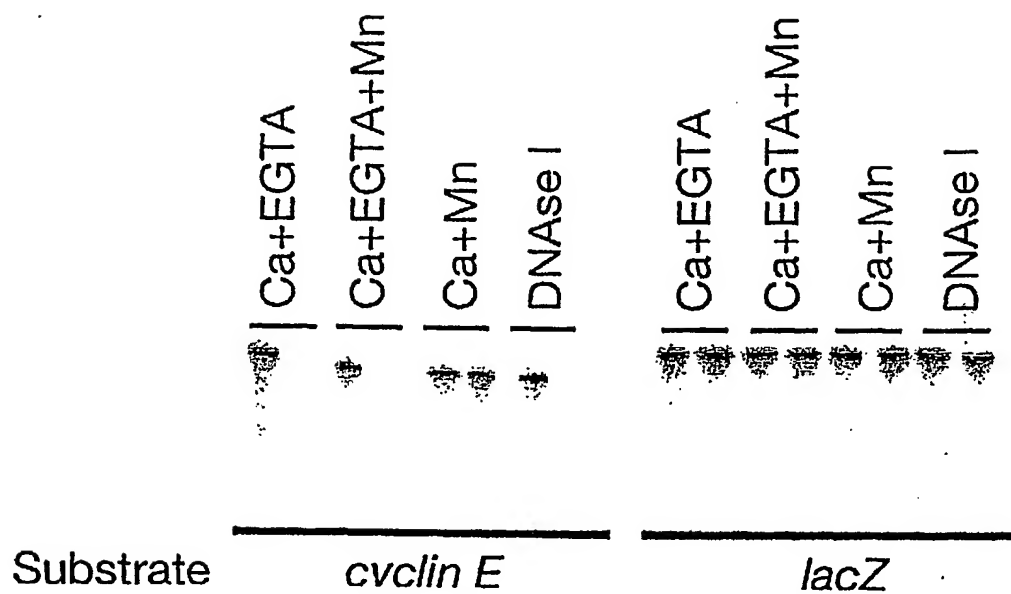
Figure 3

Figure 4

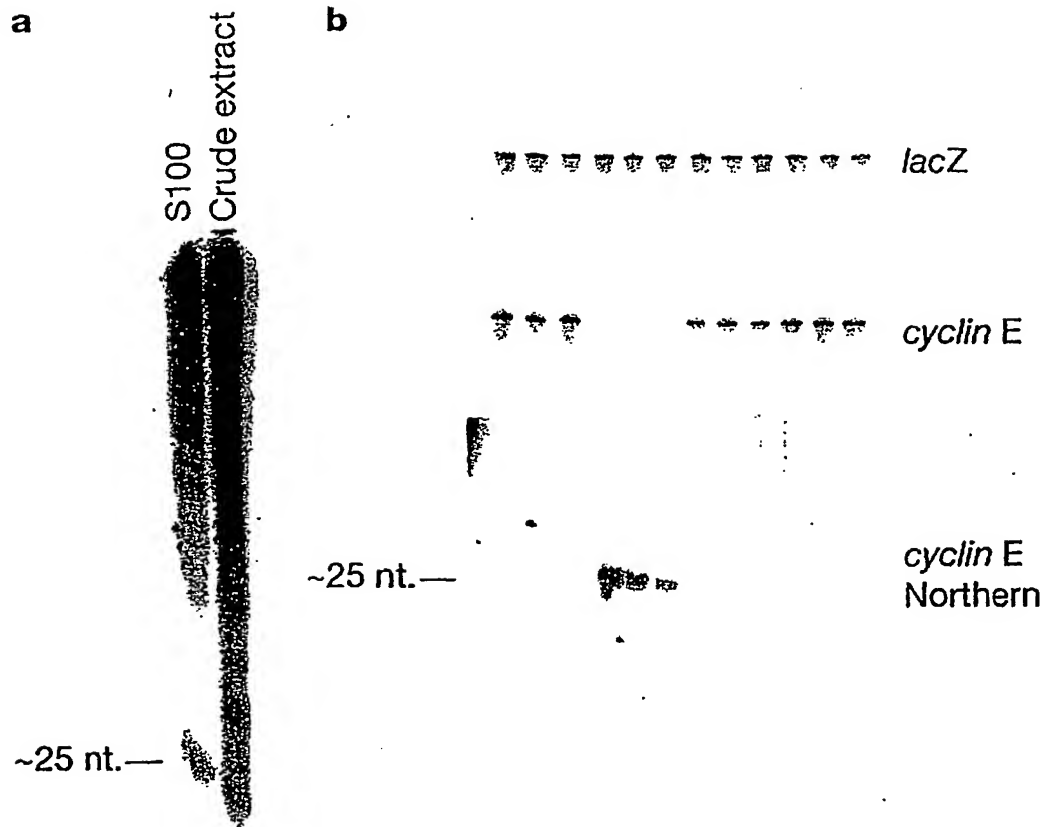


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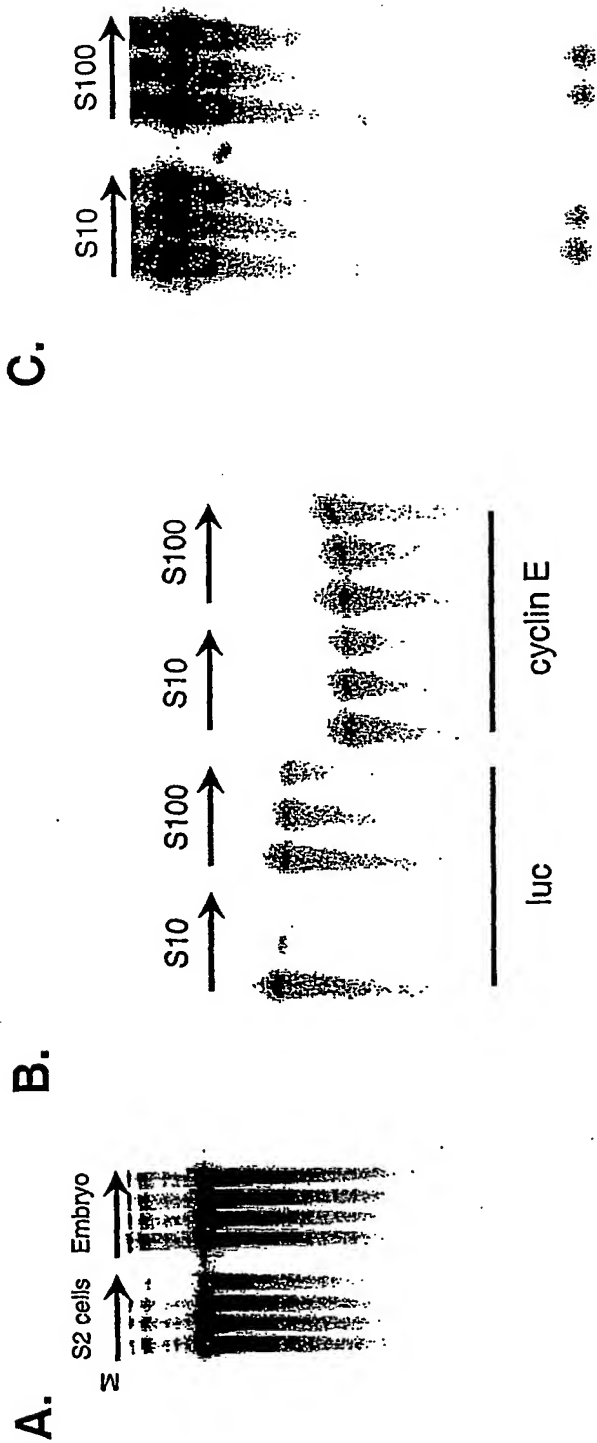
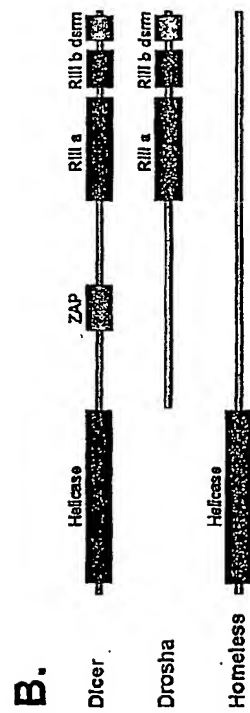
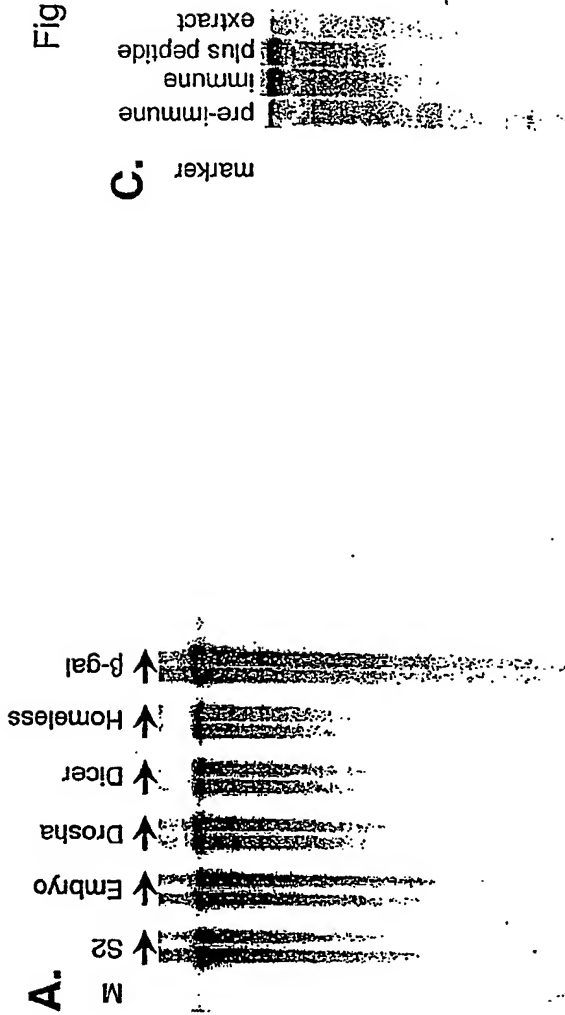


Figure 6a-c



Dicer IP
RISC
control
marker

F.

RISC - hs
RISC - ls

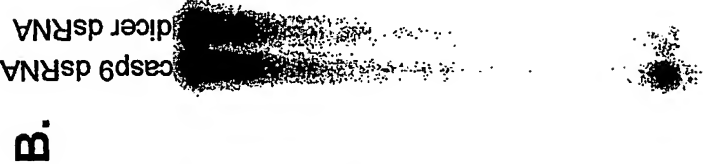
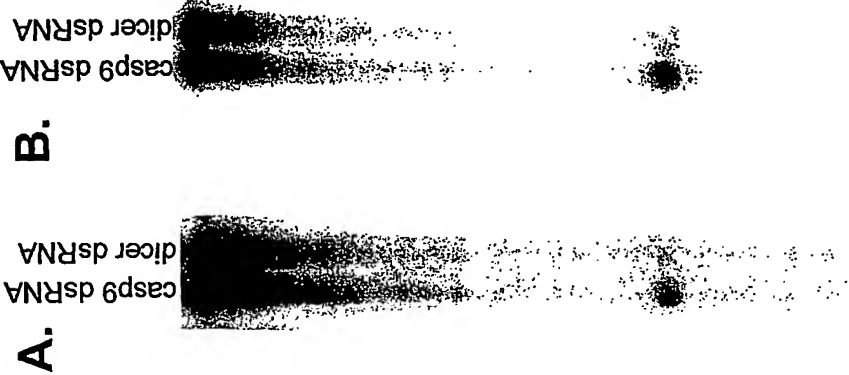
total

E.

D. IP Ext
ATP + - +

Figure 6d-f

Figure 7



C.

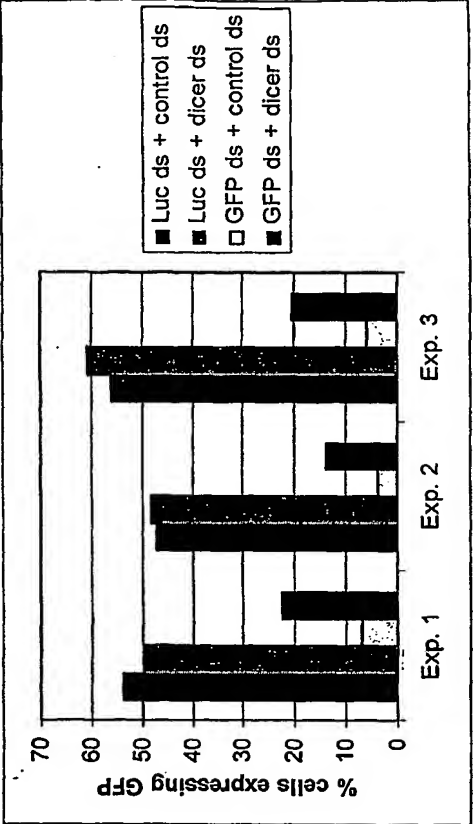


Figure 8A, B

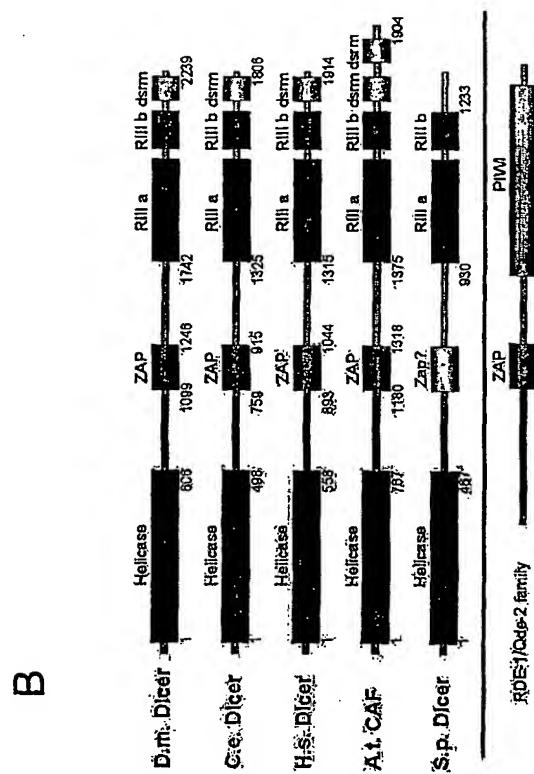


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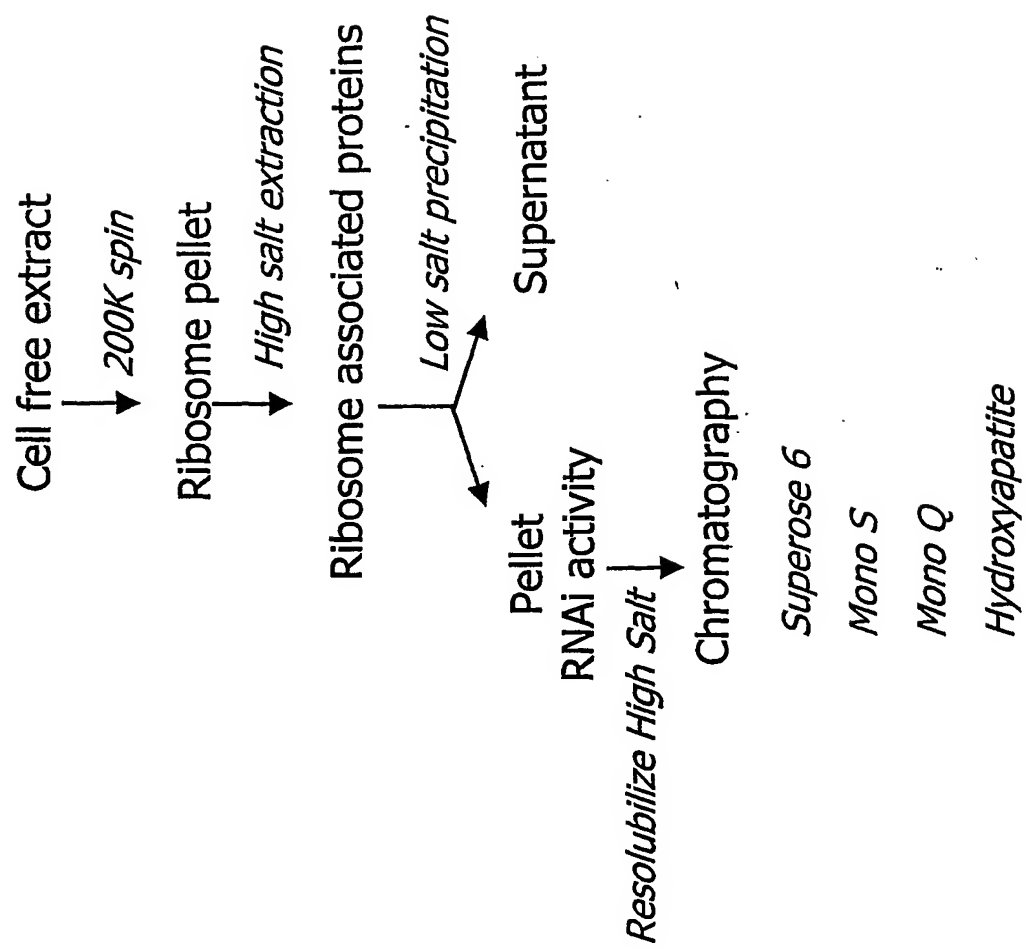


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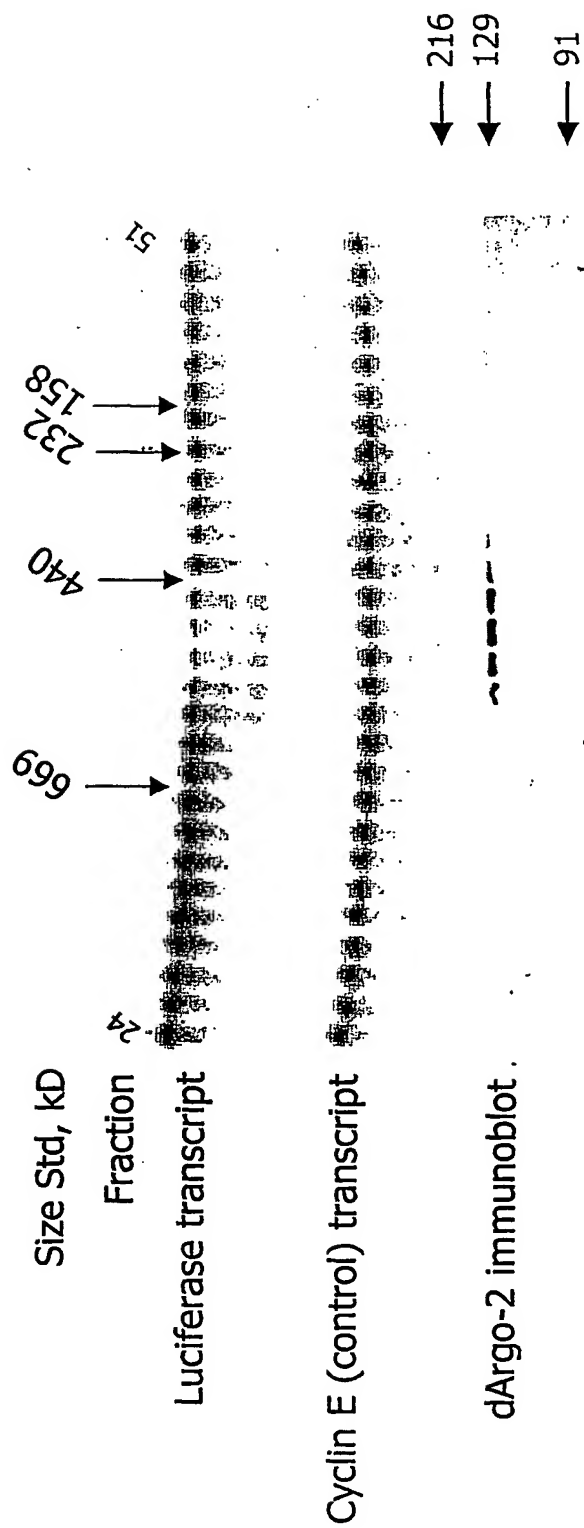


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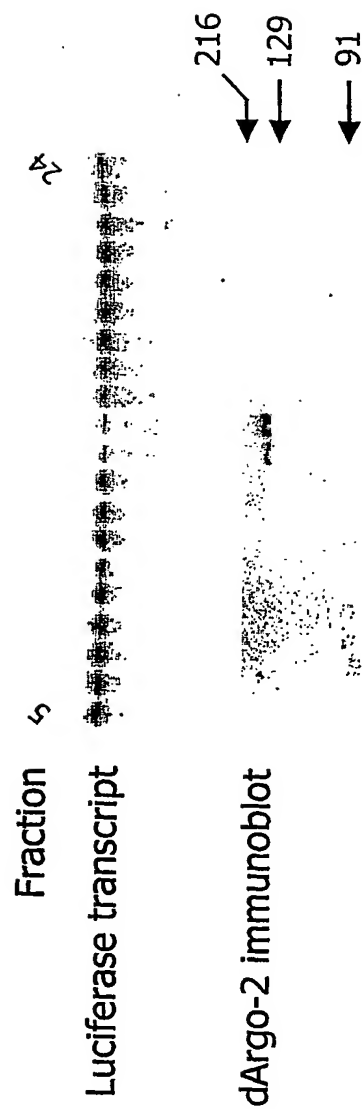


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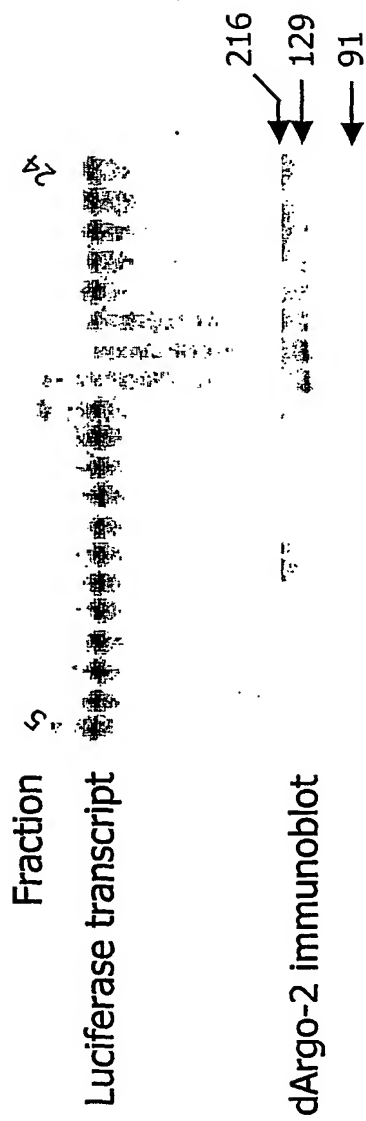
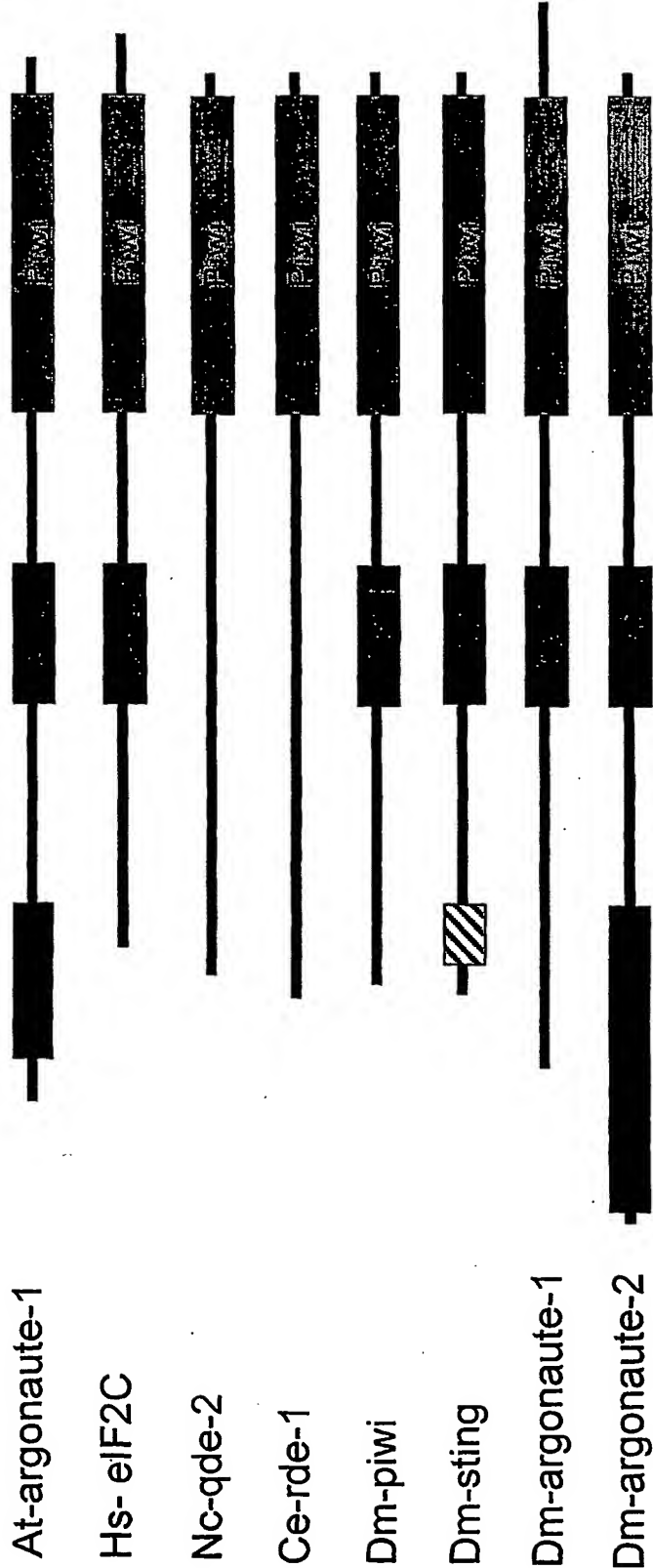


Figure 13



Figure 14



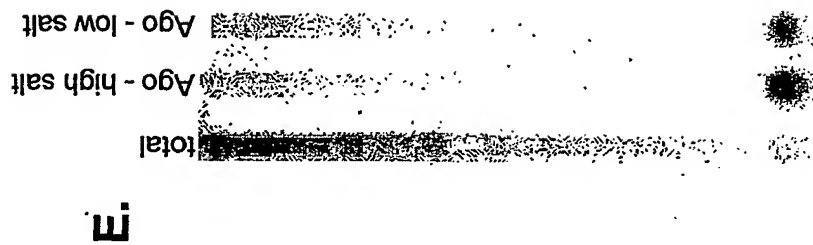


Figure 15

Figure 16

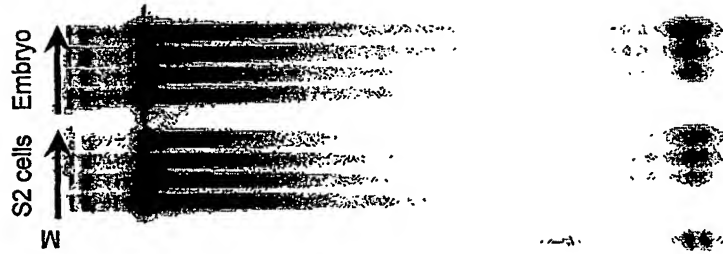


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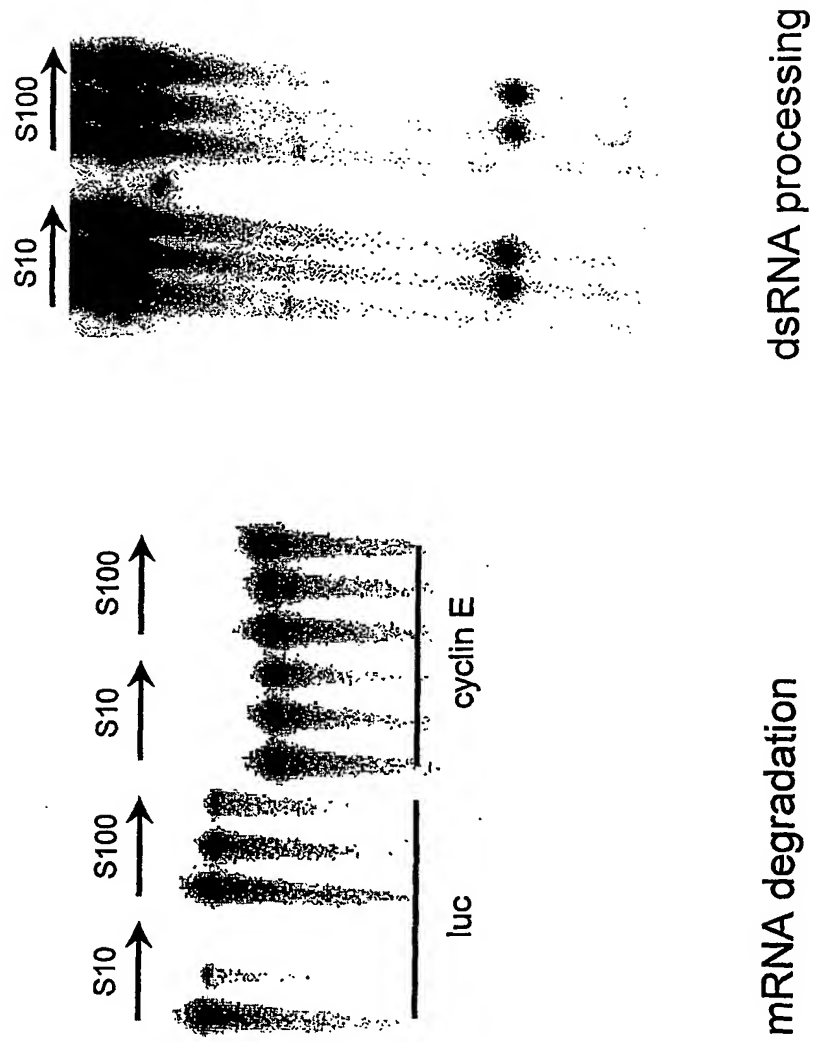
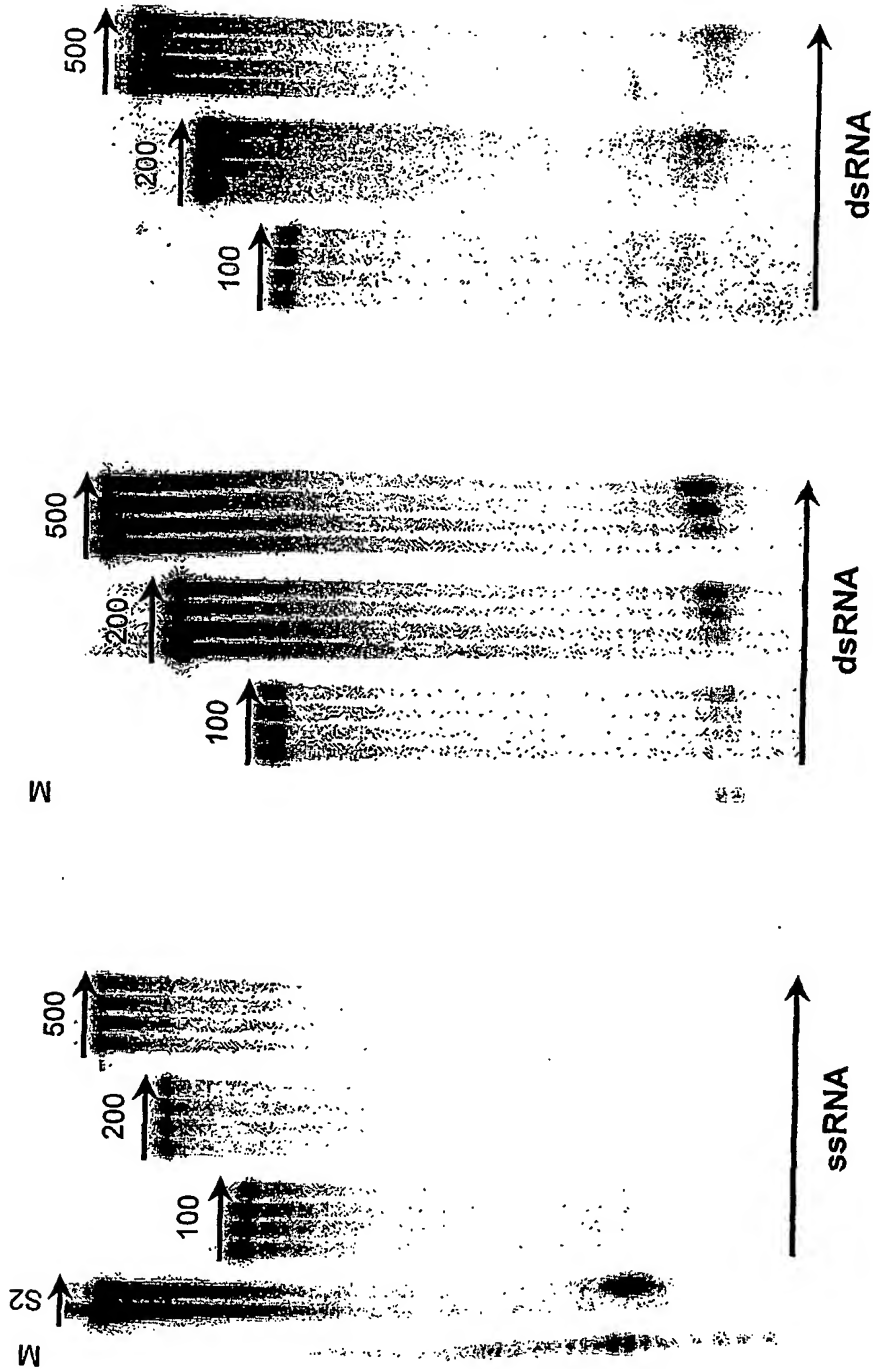


Figure 18



WO 01/68836

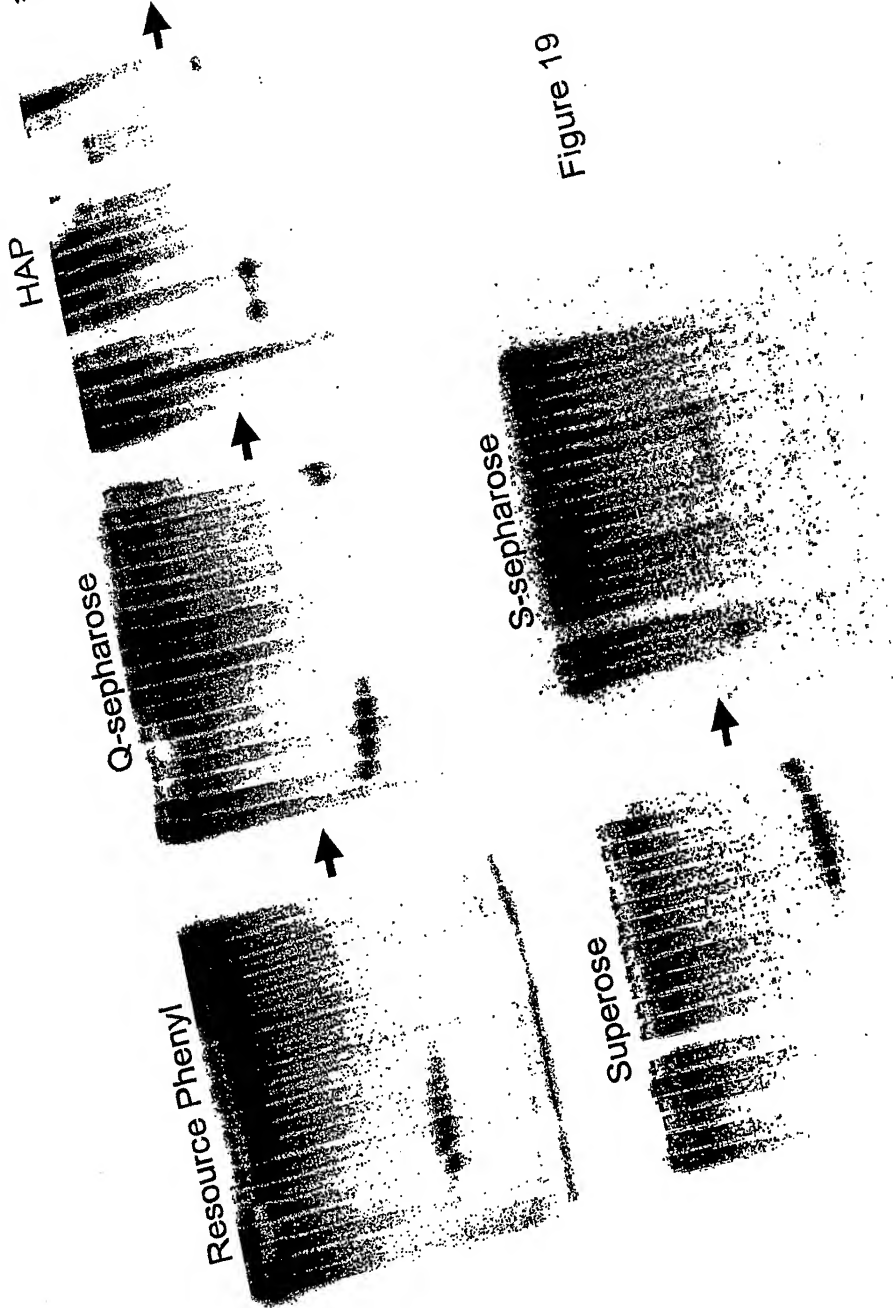


Figure 19

Purification of the 22-mer generating enzyme

Figure 20

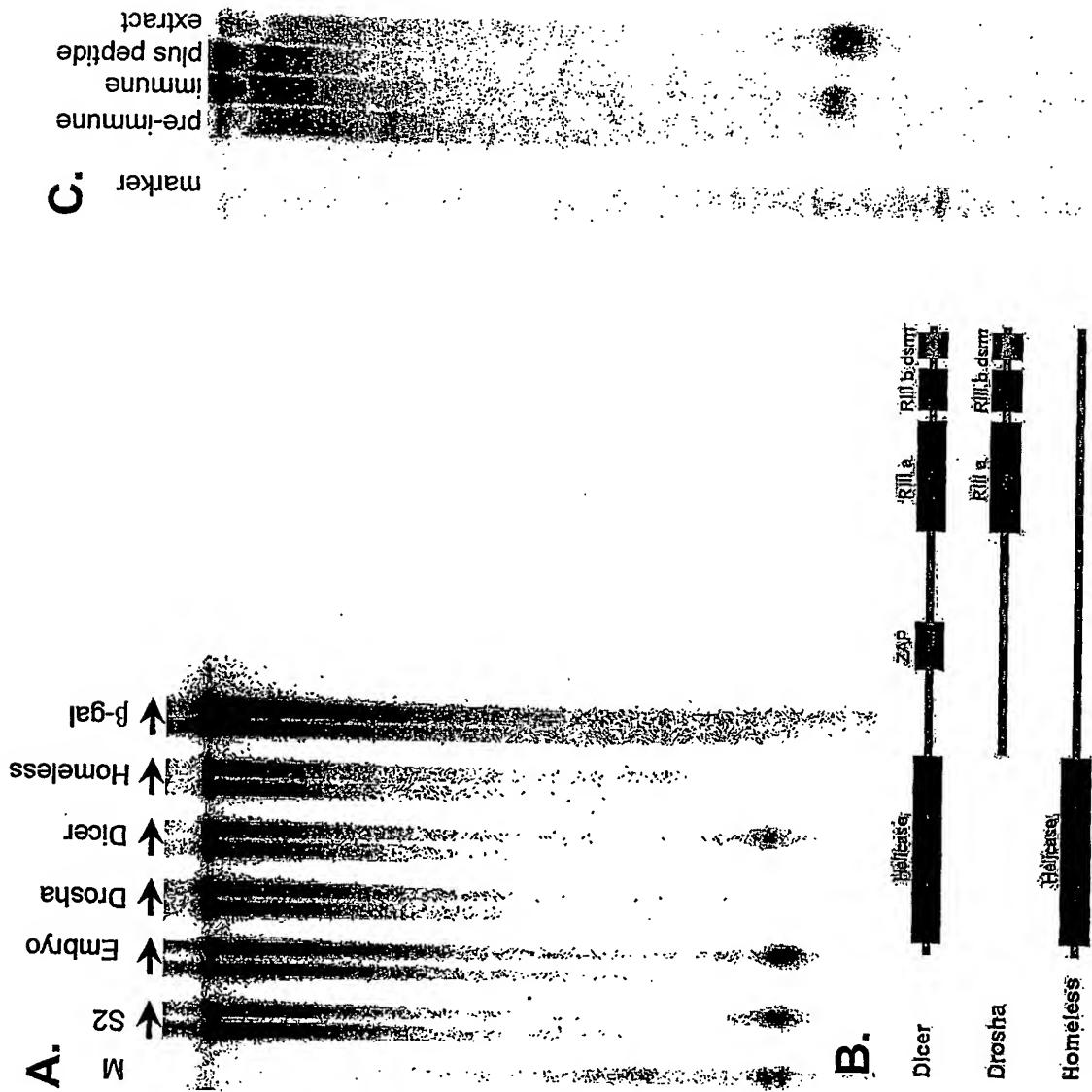
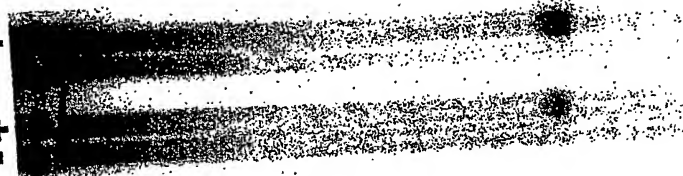


Figure 21

D.

	IP	Ext
ATP	++	++



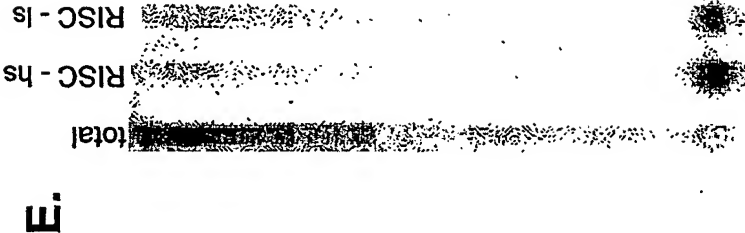


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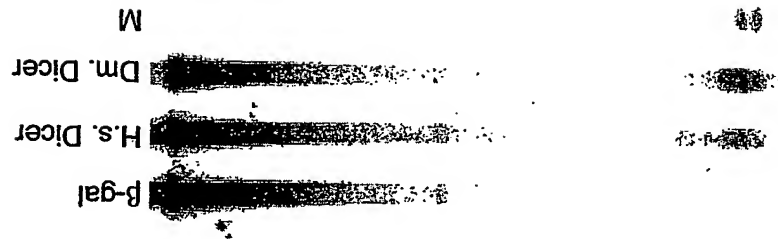


Figure 23

Figure 24

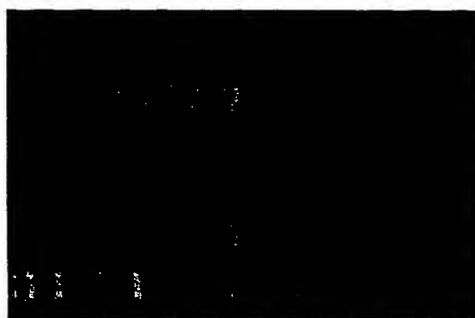
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Figure 25



S2 genomic
S2 cDNA
Library clone #7
Argo-2/p12
No template

Embryo
Adult
S2



Embryo extract
untransfected
hdicer transfected

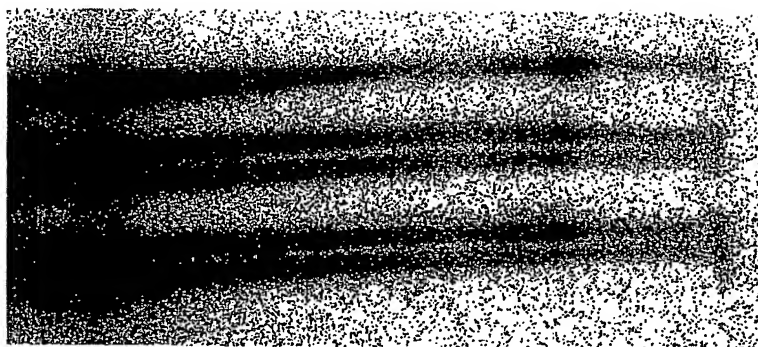
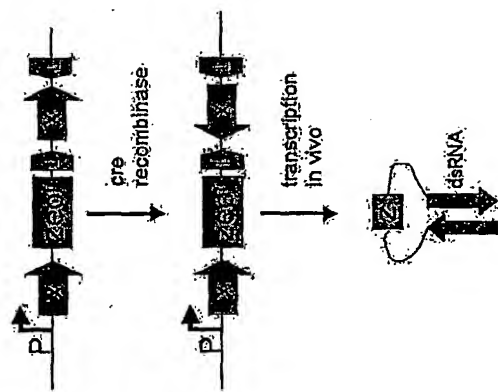


Figure 26

Figure 27



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Met Thr Pro Ala Ser Ser Pro Met Gly Pro Phe Phe Gly Leu Pro Trp

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 aac act ggc tca ggg aag aca ttt att gct agt act act cta cta aag 240
 Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
 65 70 75 80
 agc tgt ctc tat cta gat cta ggg gag act tca gct aga aat gga aaa 288
 Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
 85 90 95
 agg acg gtg ttc ttg gtc aac tct gca aac cag gtt gct caa caa gtg 336
 Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
 100 105 110
 tca gct gtc aga act cat tca gat ctc aag gtt ggg gaa tac tca aac 384
 Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
 115 120 125
 cta gaa gta aat gca tct tgg aca aaa gag aga tgg aac caa gag ttt 432
 Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
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 Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
 145 150 155 160
 ttg aaa aat ggt tac tta tca ctg tca gac att aac ctt ttg gtg ttt 528
 Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
 165 170 175

gat gag tgt cat ctt gca atc cta gac cac ccc tat cga gaa ttt atg 576
 Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
 180 185 190

aag ctc tgt gaa att tgt cca tca tgt cct cgc att ttg gga cta act 624
 Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
 195 200 205

gct tcc att tta aat ggg aaa tgg gat cca gag gat ttg gaa gaa aag 672
 Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys
 210 215 220

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 Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr
 225 230 235 240

gac ctg gtg gtc tta gac agg tat act tct cag cca tgt gag att gtg 768
 Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val
 245 250 255

gtg gat tgt gga cca ttt act gac aga agt ggg ctt tat gaa aga ctg 816
 Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu
 260 265 270

ctg atg gaa tta gaa gaa gca ctt aat ttt atc aat gat tgt aat ata 864
 Leu Met Glu Leu Glu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile
 275 280 285

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 Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
 290 295 300

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 Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
 305 310 315 320

gat aaa gta gct gga atg atg gta aga gaa cta cag aaa tac atc aaa 1008
Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
325 330 335

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His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
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355 360 365

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Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
370 375 380

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 Tyr Arg Ser Tyr Val Gln Ser Lys Gly Arg Ala Arg Ala Pro Ile Ser
 545 550 555 560

aat tat ata atg tta gcg gat aca gac aaa ata aaa agt ttt gaa gaa 1728
 Asn Tyr Ile Met Leu Ala Asp Thr Asp Lys Ile Lys Ser Phe Glu Glu
 565 570 575

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 Asp Leu Lys Thr Tyr Lys Ala Ile Glu Lys Ile Leu Arg Asn Lys Cys
 580 585 590

tcc aag tcg gtt gat act ggt gag act gac att gat cct gtc atg gat 1824
 Ser Lys Ser Val Asp Thr Gly Glu Thr Asp Ile Asp Pro Val Met Asp
 595 600 605

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610 615 620

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Pro Arg Val Thr Ile Asn Thr Ala Ile Gly His Ile Asn Arg Tyr Cys

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Ala Arg Leu Pro Ser Asp Pro Phe Thr His Leu Ala Pro Lys Cys Arg

645 650 655

acc cga gag ttg cct gat ggt aca ttt tat tca act ctt tat ctg cca 2016

Thr Arg Glu Leu Pro Asp Gly Thr Phe Tyr Ser Thr Leu Tyr Leu Pro

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Ile Asn Ser Pro Leu Arg Ala Ser Ile Val Gly Pro Pro Met Ser Cys

675 680 685

gta cga ttg gct gaa aga gtt gtc gct ctc att tgc tgt gag aaa ctg 2112

Val Arg Leu Ala Glu Arg Val Val Ala Leu Ile Cys Cys Glu Lys Leu

690 695 700

cac aaa att ggc gaa ctg gat gac cat ttg atg cca gtt ggg aaa gag 2160

His Lys Ile Gly Glu Leu Asp Asp His Leu Met Pro Val Gly Lys Glu

705 710 715 720

act gtt aaa tat gaa gag gag ctt gat ttg cat gat gaa gaa gag acc 2208

Thr Val Lys Tyr Glu Glu Glu Leu Asp Leu His Asp Glu Glu Glu Thr

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agt gtt cca gga aga cca ggt tcc acg aaa cga agg cag tgc tac cca 2256

Ser Val Pro Gly Arg Pro Gly Ser Thr Lys Arg Arg Gln Cys Tyr Pro

740 745 750

aaa gca att cca gag tgt ttg agg gat agt tat ccc aga cct gat cag 2304

Lys Ala Ile Pro Glu Cys Leu Arg Asp Ser Tyr Pro Arg Pro Asp Gln

755 760 765

ccc tgt tac ctg tat gtg ata gga atg gtt tta act aca cct tta cct 2352
Pro Cys Tyr Leu Tyr Val Ile Gly Met Val Leu Thr Thr Pro Leu Pro
770 775 780

gat gaa ctc aac ttt aga agg cgg aag ctc tat cct cct gaa gat acc 2400
Asp Glu Leu Asn Phe Arg Arg Arg Lys Leu Tyr Pro Pro Glu Asp Thr
785 790 795 800

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Thr Arg Cys Phe Gly Ile Leu Thr Ala Lys Pro Ile Pro Gln Ile Pro
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cac ttt cct gtg tac aca cgc tct gga gag gtt acc ata tcc att gag 2496
His Phe Pro Val Tyr Thr Arg Ser Gly Glu Val Thr Ile Ser Ile Glu
820 825 830

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865 870 875 880

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885 890 895

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Lys Phe Met Glu Asp Ile Glu Lys Ser Glu Ala Arg Ile Gly Ile Pro
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Ser Thr Lys Tyr Thr Lys Glu Thr Pro Phe Val Phe Lys Leu Glu Asp
915 920 925

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Tyr Gln Asp Ala Val Ile Ile Pro Arg Tyr Arg Asn Phe Asp Gln Pro
930 935 940

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His Arg Phe Tyr Val Ala Asp Val Tyr Thr Asp Leu Thr Pro Leu Ser
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Lys Phe Pro Ser Pro Glu Tyr Glu Thr Phe Ala Glu Tyr Tyr Lys Thr
965 970 975

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Lys Tyr Asn Leu Asp Leu Thr Asn Leu Asn Gln Pro Leu Leu Asp Val
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Val Cys Leu Pro Ser Ile Leu Tyr Arg Leu His Cys Leu Leu Thr
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 1070 1075 1080

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 1100 1105 1110

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 1115 1120 1125

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 Gly Ser Tyr Asp Leu Ala Asn Arg Asp Phe Cys Gln Gly Asn Gln

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Tyr Ser Ile Gln Asn Leu Tyr Ser Tyr Glu Asn Gln Pro Gln Pro

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Pro Asn Pro Gly Leu Ile Leu Gln Ala Leu Thr Leu Ser Asn Ala

1295 1300 1305

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1760 1765 1770

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1775 1780 1785

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1790 1795 1800

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1805 1810 1815

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Gly Ala Ile Tyr Met Asp Ser Gly Met Ser Leu Glu Thr Val Trp

1820 1825 1830

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1835 1840 1845

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1850 1855 1860

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1865 1870 1875

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 1895 1900 1905

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 Ser

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Val Glu Leu Leu Glu Ala Ala Leu Asp His Asn Thr Ile Val Cys Leu
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Asn Thr Gly Ser Gly Lys Thr Phe Ile Ala Ser Thr Thr Leu Leu Lys
65 70 75 80

Ser Cys Leu Tyr Leu Asp Leu Gly Glu Thr Ser Ala Arg Asn Gly Lys
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Arg Thr Val Phe Leu Val Asn Ser Ala Asn Gln Val Ala Gln Gln Val
 100 105 110

Ser Ala Val Arg Thr His Ser Asp Leu Lys Val Gly Glu Tyr Ser Asn
 115 120 125

Leu Glu Val Asn Ala Ser Trp Thr Lys Glu Arg Trp Asn Gln Glu Phe
 130 135 140

Thr Lys His Gln Val Leu Ile Met Thr Cys Tyr Val Ala Leu Asn Val
145 150 155 160

Leu Lys Asn Gly Tyr Leu Ser Leu Ser Asp Ile Asn Leu Leu Val Phe
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Asp Glu Cys His Leu Ala Ile Leu Asp His Pro Tyr Arg Glu Phe Met
 180 185 190

Lys Leu Cys Glu Ile Cys Pro Ser Cys Pro Arg Ile Leu Gly Leu Thr
 195 200 205

Ala Ser Ile Leu Asn Gly Lys Trp Asp Pro Glu Asp Leu Glu Glu Lys

210 215 220

Phe Gln Lys Leu Glu Lys Ile Leu Lys Ser Asn Ala Glu Thr Ala Thr
225 230 235 240

Asp Leu Val Val Leu Asp Arg Tyr Thr Ser Gln Pro Cys Glu Ile Val
245 250 255

Val Asp Cys Gly Pro Phe Thr Asp Arg Ser Gly Leu Tyr Glu Arg Leu
260 265 270

Leu Met Glu Leu Glu Glu Ala Leu Asn Phe Ile Asn Asp Cys Asn Ile
275 280 285

Ser Val His Ser Lys Glu Arg Asp Ser Thr Leu Ile Ser Lys Gln Ile
290 295 300

Leu Ser Asp Cys Arg Ala Val Leu Val Val Leu Gly Pro Trp Cys Ala
305 310 315 320

Asp Lys Val Ala Gly Met Met Val Arg Glu Leu Gln Lys Tyr Ile Lys
325 330 335

His Glu Gln Glu Glu Leu His Arg Lys Phe Leu Leu Phe Thr Asp Thr
340 345 350

Phe Leu Arg Lys Ile His Ala Leu Cys Glu Glu His Phe Ser Pro Ala
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Ser Leu Asp Leu Lys Phe Val Thr Pro Lys Val Ile Lys Leu Leu Glu
370 375 380

Ile Leu Arg Lys Tyr Lys Pro Tyr Glu Arg His Ser Phe Glu Ser Val
385 390 395 400

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Ser Glu Asp Asp Asp Glu Asp Glu Glu Ile Glu Glu Lys Glu Lys Pro
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 Thr Ser Glu Glu Thr Gln Leu Asp Lys Asp Ser Lys Asp Asp Lys
 1340 1345 1350

gtt gag aaa agt gct att gaa cta atc att gag gga gag gag aag 4104
 Val Glu Lys Ser Ala Ile Glu Leu Ile Ile Glu Gly Glu Glu Lys
 1355 1360 1365

ctg caa gag gct gat gac ttc att gag ata ggc act tgg tca aac 4149
 Leu Gln Glu Ala Asp Asp Phe Ile Glu Ile Gly Thr Trp Ser Asn
 1370 1375 1380

gat atg gcc gac gat ata gct agt ttt aac caa gaa gac gac gac 4194
 Asp Met Ala Asp Asp Ile Ala Ser Phe Asn Gln Glu Asp Asp Asp
 1385 1390 1395

gag gat gac gcc ttc cat ctc cca gtt tta ccg gca aac gtt aag 4239

Glu Asp Asp Ala Phe His Leu Pro Val Leu Pro Ala Asn Val Lys
 1400 1405 1410

ttc tgt gat cag caa acg cgc tac ggt tcg ccc aca ttt tgg gat 4284
 Phe Cys Asp Gln Gln Thr Arg Tyr Gly Ser Pro Thr Phe Trp Asp
 1415 1420 1425

gtg agc aat ggc gaa agc ggc ttc aag ggt cca aag agc agt cag 4329
 Val Ser Asn Gly Glu Ser Gly Phe Lys Gly Pro Lys Ser Ser Gln
 1430 1435 1440

aat aag cag ggt ggc aag ggc aaa gca aag ggt ccg gca aag ccc 4374
 Asn Lys Gln Gly Gly Lys Gly Lys Ala Lys Gly Pro Ala Lys Pro
 1445 1450 1455

aca ttt aac tat tat gac tcg gac aat tcg ctg ggt tcc agc tac 4419
 Thr Phe Asn Tyr Tyr Asp Ser Asp Asn Ser Leu Gly Ser Ser Tyr
 1460 1465 1470

gat gac gac gat aac gca ggt ccg ctc aat tac atg cat cac aac 4464
 Asp Asp Asp Asp Asn Ala Gly Pro Leu Asn Tyr Met His His Asn
 1475 1480 1485

tac agt tcg gat gac gac gat gtg gca gat gat atc gat gcg gga 4509
 Tyr Ser Ser Asp Asp Asp Asp Val Ala Asp Asp Ile Asp Ala Gly
 1490 1495 1500

cgc att gcg ttc acc tcc aag aat gaa gcg gag act att gaa acc 4554
 Arg Ile Ala Phe Thr Ser Lys Asn Glu Ala Glu Thr Ile Glu Thr
 1505 1510 1515

gca cag gaa gtg gaa aag cgc cag aag cag ctg tcc atc atc cag 4599
 Ala Gln Glu Val Glu Lys Arg Gln Lys Gln Leu Ser Ile Ile Gln
 1520 1525 1530

gcg acc aat gct aac gag cgg cag tat cag cag aca aag aac ctg 4644
 Ala Thr Asn Ala Asn Glu Arg Gln Tyr Gln Gln Thr Lys Asn Leu

1535 1540 1545

ctc att gga ttc aat ttt aag cat gag gac cag aag gaa cct gcc 4689

Leu Ile Gly Phe Asn Phe Lys His Glu Asp Gln Lys Glu Pro Ala

1550 1555 1560

act ata aga tat gaa gaa tcc ata gct aag ctc aaa acg gaa ata 4734

Thr Ile Arg Tyr Glu Glu Ser Ile Ala Lys Leu Lys Thr Glu Ile

1565 1570 1575

gaa tcc ggc ggc atg ttg gtg ccg cac gac cag cag ttg gtt cta 4779

Glu Ser Gly Gly Met Leu Val Pro His Asp Gln Gln Leu Val Leu

1580 1585 1590

aaa aga agt gat gcc gct gag gct cag gtt gca aag gta tcg atg 4824

Lys Arg Ser Asp Ala Ala Glu Ala Gln Val Ala Lys Val Ser Met

1595 1600 1605

atg gag cta ttg aag cag ctg ctg ccg tat gta aat gaa gat gtg 4869

Met Glu Leu Leu Lys Gln Leu Leu Pro Tyr Val Asn Glu Asp Val

1610 1615 1620

ctg gcc aaa aag ctg ggt gat agg cgc gag ctt ctg ctg tcg gat 4914

Leu Ala Lys Lys Leu Gly Asp Arg Arg Glu Leu Leu Leu Ser Asp

1625 1630 1635

ttg gta gag cta aat gca gat tgg gta gcg cga cat gag cag gag 4959

Leu Val Glu Leu Asn Ala Asp Trp Val Ala Arg His Glu Gln Glu

1640 1645 1650

acc tac aat gta atg gga tgc gga gat agt ttt gac aac tat aac 5004

Thr Tyr Asn Val Met Gly Cys Gly Asp Ser Phe Asp Asn Tyr Asn

1655 1660 1665

gat cat cat cgg ctg aac ttg gat gaa aag caa ctg aaa ctg caa 5049

Asp His His Arg Leu Asn Leu Asp Glu Lys Gln Leu Lys Leu Gln

1670 1675 1680

tac gaa cga att gaa att gag cca cct act tcc acg aag gcc ata 5094

Tyr Glu Arg Ile Glu Ile Glu Pro Pro Thr Ser Thr Lys Ala Ile

1685 1690 1695

acc tca gcc ata tta cca gct ggc ttc agt ttc gat cga caa ccg 5139

Thr Ser Ala Ile Leu Pro Ala Gly Phe Ser Phe Asp Arg Gln Pro

1700 1705 1710

gat cta gtg ggc cat cca gga ccc agt ccc agc atc att ttg caa 5184

Asp Leu Val Gly His Pro Gly Pro Ser Pro Ser Ile Ile Leu Gln

1715 1720 1725

gcc ctc aca atg tcc aat gct aac gat ggc atc aat ctg gag cga 5229

Ala Leu Thr Met Ser Asn Ala Asn Asp Gly Ile Asn Leu Glu Arg

1730 1735 1740

ctg gag aca att gga gat tcc ttt cta aag tat gcc att acc acc 5274

Leu Glu Thr Ile Gly Asp Ser Phe Leu Lys Tyr Ala Ile Thr Thr

1745 1750 1755

tac ttg tac atc acc tac gag aat gtg cac gag gga aaa cta agt 5319

Tyr Leu Tyr Ile Thr Tyr Glu Asn Val His Glu Gly Lys Leu Ser

1760 1765 1770

cac ctg cgc tcc aag cag gtt gcc aat ctc aat ctc tat cgt ctg 5364

His Leu Arg Ser Lys Gln Val Ala Asn Leu Asn Leu Tyr Arg Leu

1775 1780 1785

ggc aga cgt aag aga ctg ggt gaa tat atg ata gcc act aaa ttc 5409

Gly Arg Arg Lys Arg Leu Gly Glu Tyr Met Ile Ala Thr Lys Phe

1790 1795 1800

gag ccg cac gac aat tgg ctg cca ccc tgc tac tac gtg cca aag 5454

Glu Pro His Asp Asn Trp Leu Pro Pro Cys Tyr Tyr Val Pro Lys

1805 1810 1815

gag cta gag aag gcg ctc atc gag gcg aag atc ccc act cac cat 5499
 Glu Leu Glu Lys Ala Leu Ile Glu Ala Lys Ile Pro Thr His His
 1820 1825 1830

tgg aag ctg gcc gat ctg cta gac att aag aac cta agc agt gtg 5544
 Trp Lys Leu Ala Asp Leu Leu Asp Ile Lys Asn Leu Ser Ser Val
 1835 1840 1845

caa atc tgc gag atg gtt cgc gaa aaa gcc gat gcc ctg ggc ttg 5589
 Gln Ile Cys Glu Met Val Arg Glu Lys Ala Asp Ala Leu Gly Leu
 1850 1855 1860

gag cag aat ggg ggt gcc caa aat gga caa ctt gac gac tcc aat 5634
 Glu Gln Asn Gly Gly Ala Gln Asn Gly Gln Leu Asp Asp Ser Asn
 1865 1870 1875

gat agc tgc aat gat ttt agc tgt ttt att ccc tac aac ctt gtt 5679
 Asp Ser Cys Asn Asp Phe Ser Cys Phe Ile Pro Tyr Asn Leu Val
 1880 1885 1890

tgc caa cac agc att ccg gat aag tct att gcc gat tgc gtc gaa 5724
 Ser Gln His Ser Ile Pro Asp Lys Ser Ile Ala Asp Cys Val Glu
 1895 1900 1905

gcc ctc att gga gcc tat ctc att gag tgc gga ccc cga ggg gct 5769
 Ala Leu Ile Gly Ala Tyr Leu Ile Glu Cys Gly Pro Arg Gly Ala
 1910 1915 1920

tta ctc ttt atg gcc tgg ctg ggc gtg aga gtg ctc cct atc aca 5814
 Leu Leu Phe Met Ala Trp Leu Gly Val Arg Val Leu Pro Ile Thr
 1925 1930 1935

agg cag ttg gac ggg ggt aac cag gag caa cga ata ccc ggt agc 5859
 Arg Gln Leu Asp Gly Gly Asn Gln Glu Gln Arg Ile Pro Gly Ser
 1940 1945 1950

aca aaa ccg aat gcc gaa aat gtg gtc acc gtt tac ggt gca tgg 5904

Thr Lys Pro Asn Ala Glu Asn Val Val Thr Val Tyr Gly Ala Trp
 1955 1960 1965

ccc acg ccg cgt agt cca ctg ctg cac ttt gct cca aat gct acg 5949
 Pro Thr Pro Arg Ser Pro Leu Leu His Phe Ala Pro Asn Ala Thr
 1970 1975 1980

gag gag ctg gac cag tta cta agc ggc ttt gag gag ttt gag gag 5994
 Glu Glu Leu Asp Gln Leu Leu Ser Gly Phe Glu Glu Phe Glu Glu
 1985 1990 1995

agt ttg gga tac aag ttc cgg gat cgg tcg tac ctg ttg caa gcc 6039
 Ser Leu Gly Tyr Lys Phe Arg Asp Arg Ser Tyr Leu Leu Gln Ala
 2000 2005 2010

atg aca cat gcc agt tac acg ccc aat cga ttg acg gat tgc tat 6084
 Met Thr His Ala Ser Tyr Thr Pro Asn Arg Leu Thr Asp Cys Tyr
 2015 2020 2025

cag cgt ctg gag ttc ctg ggc gat gct gtt cta gat tac ctc att 6129
 Gln Arg Leu Glu Phe Leu Gly Asp Ala Val Leu Asp Tyr Leu Ile
 2030 2035 2040

acg cgg cat tta tac gaa gat ccc cgc cag cat tct cca ggc gca 6174
 Thr Arg His Leu Tyr Glu Asp Pro Arg Gln His Ser Pro Gly Ala
 2045 2050 2055

tta acg gat ttg cgg tca gca ctg gtg aat aat aca ata ttc gcc 6219
 Leu Thr Asp Leu Arg Ser Ala Leu Val Asn Asn Thr Ile Phe Ala
 2060 2065 2070

tcc ctg gct gtt cgc cat ggc ttc cac aag ttc ttc cgg cac ctc 6264
 Ser Leu Ala Val Arg His Gly Phe His Lys Phe Phe Arg His Leu
 2075 2080 2085

tcg ccg ggc ctt aac gat gtg att gac cgt ttt gtg cgg atc cag 6309
 Ser Pro Gly Leu Asn Asp Val Ile Asp Arg Phe Val Arg Ile Gln